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ADIPOSE TISSUE INFLAMMATION, LIVER FAT AND INSULIN RESISTANCE IN HUMANS

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ACADEMIC DISSERTATION

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ABBREVIATIONS AND DEFINITIONS

ACTB	β-actin
ADAM8	A disintegrin and metallopeptidase domain 8
AIC	The Akaike information criterion
ALT	Alanine aminotransferase
Apo	Apolipoprotein
ASP	Acylation stimulating protein
AST	Aspartate aminotransferase
ATGL	Adipose triacylglycerol lipase
BMI	Body mass index
CCR2	C-C motif chemokine receptor 2
cDNA	Complementary deoxyribonucleic acid
CD68	Cluster of differentiation 68
CLS	Crown-like structure
CoA	Coenzyme A
CRP	C-reactive protein
CVD	Cardiovascular disease(s)
DAG	Diacylglycerol(s)
DEXA	Dual-energy X-ray absorptiometry
DNL	*De novo* lipogenesis
DZ	Dizygotic
EGP	Endogenous glucose production
ELISA	Enzyme-linked immunosorbent assay
EMR1	Epidermal growth factor module-containing mucin-like hormone receptor 1
ER	Endoplasmic reticulum
FA	Fatty acid(s)
FFA	Free fatty acid(s) (denotes *circulating* free fatty acids)
FFM	Fat free mass
GLUT	Facilitated glucose transporter
GM-CSF	Granylocyte-macrophage colony-stimulating factor
GNG	Gluconeogenesis
γGT	γ-glutamyl transferase
G6P	Glucose-6-phosphate
HbA_{1c}	Glycosylated hemoglobin A_{1c}
HDL	High-density lipoprotein
HSL	Hormone sensitive lipase
IGT	Impaired glucose tolerance
IKK-β	Inhibitor of IκB
IL	Interleukin
IMCL	Intramyocellular lipid
IRS	Insulin-receptor substrate protein
ITGAM	Integrin α M
JNK	C-Jun amino-terminal kinase
KO	Knock-out
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1

M-CSF	Macrophage colony-stimulating factor
MIF	Macrophage migration inhibitory factor
MGL	Monoacylglycerol lipase
MetS	Metabolic syndrome
MIP-1α	Macrophage inflammatory protein 1 α
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
mRNA	Messenger ribonucleic acid
MZ	Monozygotic
M-value	Whole body insulin sensitivity
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NFκB	Nuclear factor κ B
OPN	Osteopontin
PAI-1	Plasminogen activator inhibitor 1
PDH	Pyruvate dehydrogenase
PET	Positron emission tomography
PFK	Phosphofructokinase
PGC-1α	Peroxisome proliferator-activated receptor coactivator 1 α
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PNPLA3	Patatin-like phospholipase domain-containing 3
PPAR	Peroxisome proliferator-activated receptor
RBP-4	Retinol binding protein 4
RPLP0	Ribosomal protein large P0
RT-PCR	Real-time polymerase chain reaction
SMPD	Sphingomyelinase (protein is also known as SMase)
SNP	Single nucleotide polymorphism
SPT	Serine palmitoyltransferase
SVF	Stromal vascular fraction
TAG	Triacylglycerol(s)
TBP	TATA-box binding protein
TGF-β1	Transforming growth factor β 1
TNFα	Tumor necrosis factor α
TSP-1	Thrombospondin 1
T2DM	Type 2 diabetes mellitus
VLDL	Very low-density lipoprotein
W/H	Waist-to-hip ratio
WT	Wild-type
11β-HSD-1	11β-hydroxysteroid dehydrogenase type 1
^{1}H-MRS	Proton magnetic resonance spectroscopy
3T3-L1	Name of a murine preadipocyte cell line used to study adipocyte metabolism
95 % CI	95 % confidence intervals

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by their Roman numerals:

I) Westerbacka J, Cornér A, Kannisto K, Kolak M, **Makkonen J**, Korsheninnikova E, Nyman T, Hamsten A, Fisher RM, Yki-Järvinen H. Acute *in vivo* effects of insulin on gene expression in adipose tissue in insulin-resistant and insulin-sensitive subjects. Diabetologia, 49:132-140, 2006.

II) Westerbacka J, Cornér A, Kolak M, **Makkonen J**, Turpeinen U, Hamsten A, Fisher RM, Yki-Järvinen H. Insulin regulation of MCP-1 in human adipose tissue of obese and lean women. American Journal of Physiology: Endocrinology and Metabolism, 294:E841-E845, 2008.

III) **Makkonen J**, Westerbacka J, Kolak M, Sutinen J, Cornér A, Hamsten A, Fisher RM, Yki-Järvinen H. Increased expression of the macrophage markers and of 11β-HSD-1 in subcutaneous adipose tissue, but not in cultured monocyte-derived macrophages, is associated with liver fat in human obesity. International Journal of Obesity, 31:1617-1625, 2007.

IV) Kolak M, Westerbacka J, Velagapudi VR, Wågsäter D, Yetukuri L, **Makkonen J**, Rissanen A, Häkkinen A-M, Lindell M, Bergholm R, Hamsten A, Eriksson P, Fisher RM, Orešič M, Yki-Järvinen H. Adipose tissue inflammation and increased ceramide content characterize subjects with high liver fat content independent of obesity. Diabetes, 56:1960-1968, 2007.

V) **Makkonen J**, Pietiläinen KH, Rissanen A, Kaprio J, Yki-Järvinen H. Genetic factors contribute to variation in serum alanine aminotransferase activity independent of obesity and alcohol: A study in monozygotic and dizygotic twins. Journal of Hepatology, 50:1035-1042, 2009.

In addition, some unpublished data have been presented.

The publication IV has been included in the thesis for doctoral degree by Maria Kolak at Karolinska Institutet (Stockholm, Sweden, 2008)

ABSTRACT

Obesity is closely associated with insulin resistance, which is a pathophysiologic condition contributing to the important co-morbidities of obesity, such as the metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM). However, not all obese subjects develop insulin resistance. In obese subjects, adipose tissue is characterized by inflammation with macrophage infiltration and increased expression of signalling peptides (adipocytokines) counteracting insulin action, as well as decreased expression of insulin-sensitizing molecules. Increased liver fat accumulation, without excessive alcohol consumption or other known causes of liver disease, is also associated with obesity and insulin resistance. The latter condition is defined as non-alcoholic fatty liver disease (NAFLD). It is unknown whether and how insulin resistance is associated with altered expression of adipocytokines in adipose tissue, and whether adipose tissue inflammation and NAFLD coexist independent of obesity. Genetic factors could explain variation in liver fat independent of obesity but the heritability of NAFLD is unknown.

The present studies were undertaken to determine whether acute regulation of adipocytokine expression by insulin in adipose tissue is altered in obesity. The studies also aimed to investigate the relationship between adipose tissue inflammation and liver fat content independent of obesity. In addition, heritability of serum alanine aminotransferase (ALT) activity, a surrogate marker of liver fat, was assessed.

A total of 55 normal-weight and obese non-diabetic and healthy volunteers were recruited for studies I-IV. For study V, 313 healthy individual twins were recruited from a large population-based cohort. In studies I and II, subcutaneous adipose tissue biopsies were obtained for measurement of gene expression before and during 6 hours of euglycemic hyperinsulinemia. In studies III and IV, liver fat content was measured by proton magnetic resonance spectroscopy (^{1}H-MRS), and adipose tissue inflammation was assessed by gene expression, immunohistochemistry and lipidomics analysis. In study V, genetic factors contributing to serum ALT activity were determined by statistical heritability modeling.

Studies I and II demonstrated that during *in vivo* insulin infusion the expression of genes related to insulin sensitivity remains unchanged, while the expression of genes related to insulin resistance and inflammation increases in obese and insulin-resistant subjects compared to insulin-sensitive subjects. Studies III and IV demonstrated that adipose tissue inflammation is associated with liver fat content independent of obesity. Adipose tissue of subjects with high liver fat content is characterized by increased number of macrophages and increased expression of inflammatory genes, as well as by increased concentrations of ceramides and long-chain triacylglycerols (TAG) when compared to equally obese subjects with normal liver fat. Study V verified significant heritability for serum ALT activity.

In conclusion, the effects of insulin infusion on adipose tissue gene expression in obese and insulin-resistant subjects are not only characterized by hyporesponse of insulin sensitivity

genes but also by hyperresponse of insulin resistance and inflammatory genes. This suggests that in obesity, the impaired insulin action contributes or self-perpetuates alterations in adipocytokine expression in adipose tissue. Adipose tissue inflammation, defined by increased infiltration of macrophages and expression of inflammatory genes, is increased in subjects with high liver fat compared to equally obese subjects with normal liver fat content. Ceramides, the putative mediators of insulin resistance, are also the most upregulated lipid species in lipidomics analysis of adipose tissue in subjects with high liver fat. In addition, genetic factors contribute significantly to variation in serum ALT activity, a surrogate marker of liver fat. These data imply that adipose tissue inflammation and increased liver fat content are closely interrelated, and determine insulin resistance even independent of obesity.

INTRODUCTION

Obesity is closely associated with increasing insulin resistance, which is an important pathophysiologic condition contributing to clinically important co-morbidities of obesity, such as the MetS and T2DM (Kahn and Flier 2000). However, not all obese subjects are insulin-resistant or develop the MetS, and the reasons for this are poorly understood.

Adipose tissue is not merely a passive depot for storage of excess energy mainly in the form of TAG, but also a metabolically active endocrine organ secreting numerous peptides called adipocytokines (Kershaw and Flier 2004). Adipose tissue has been shown to be inflamed and infiltrated by inflammatory macrophages in obese mice and humans compared to lean counterparts (Weisberg et al. 2003, Xu et al. 2003b). The expression and production of proinflammatory adipocytokines, such as tumor necrosis factor α (TNFα), interleukin 6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1), by adipose tissue have been shown to be increased in obese and insulin-resistant subjects. Adipose tissue inflammation and adipocytokines may cause both local and systemic insulin resistance. On the other hand, *in vitro* studies and animal models have suggested that regulation of some genes may retain sensitivity to insulin action even under insulin-resistant states (Sartipy and Loskutoff 2003). To date, the triggers of adipose tissue inflammation and mechanisms by which adipose tissue inflammation relates to whole body insulin resistance in humans are incompletely understood.

Accumulation of excess fat in the liver (i.e. steatosis), not caused by alcohol or other known causes of liver disease, is defined as NAFLD (Neuschwander-Tetri and Caldwell 2003). The fatty liver in NAFLD is insulin-resistant and produces excess amounts of both glucose and very low-density lipoproteins (VLDL) (Ryysy et al. 2000) leading to hyperglycemia, hyperinsulinemia and hypertriglyceridemia (Seppälä-Lindroos et al. 2002, Adiels et al. 2006). NAFLD is closely related to all components of the MetS independent of obesity (Kotronen and Yki-Järvinen 2008). The increased release of free fatty acids (FFA) and uncontrolled production of adipocytokines by inflamed and insulin-resistant adipose tissue may contribute to liver fat content. However, it is unknown whether adipose tissue inflammation and fat accumulation in the liver are related independent of obesity. Concerning causes of liver fat accumulation in NAFLD, acquired and environmental factors, such as changes in body weight, have been shown to contribute. Studies assessing the effects of genetic factors on variation in liver fat content are limited.

The present studies aimed to assess the acute effects of insulin on adipose tissue expression of genes associated with insulin sensitivity, insulin resistance and inflammation in healthy insulin-sensitive and insulin-resistant subjects. The aim was also to determine whether adipose tissue inflammation is related to liver fat content independent of obesity and whether there are differences in infiltration of macrophages and gene expression in adipose tissue between equally obese subjects with normal or high liver fat content. In addition, the heritability of serum ALT activity, a surrogate marker of liver fat, was determined in healthy young adult twins.

REVIEW OF THE LITERATURE

1. Lipid and glucose metabolism under normal physiological conditions

1.1. Adipose tissue

Adipose tissue is, in addition to skeletal muscle and the liver, one of the three main insulin target tissues (Yki-Järvinen 1993). The key function of adipose tissue is the storage of excess energy in the form of triacylglycerols (TAG), and when needed, mobilization of TAG for delivery to other tissues, such as skeletal muscle and the liver, for energy production. Depending on the nutritional state, TAG stores are increased or mobilized. Insulin is the key regulator of metabolism of fatty acids (FA) in adipose tissue (Duncan et al. 2007). In addition, adipose tissue functions as an autocrine, paracrine and endocrine organ secreting adipocytokines that mediate inflammation and regulate insulin action (Kershaw and Flier 2004).

1.1.1. Triacylglycerol storage and release

The fasting state. In the fasting state (i.e. in the postabsorptive phase), circulating concentrations of glucose and insulin are low. Lipoprotein lipase (LPL) is an enzyme located on the luminal side of endothelial cells of adipose tissue capillaries catalyzing lipolysis of VLDL and chylomicrons (Scow et al. 1980). During this intravascular lipolysis, part of the hydrolyzed FFA are released directly into the blood stream (Frayn et al. 1995), a phenomenon called FFA spillover (Miles and Nelson 2007, Ruge et al. 2009). Activity of LPL is regulated by insulin and is low in the fasting state. Low insulin concentration, on the other hand, allows hormone sensitive lipase (HSL), the major intracellular lipolytic enzyme inside adipocytes (Frayn et al. 2003), to hydrolyze adipocyte TAG stores together with adipose triacylglycerol lipase (ATGL). Part of the FA released from intracellular TAG stores is rapidly re-esterified within adipocytes, but this activity is low during fasting (Frayn et al. 1994). Thus, there is a net release of FFA from adipose tissue in the fasting state. Glycerol kinase activity that converts free glycerol into glycerol-3-phosphate for synthesis of TAG, is low to negligible in human adipose tissue. Free glycerol is released by adipose tissue and mainly utilized by the liver and kidneys that possess glycerol kinase activity (Guo and Jensen 1999).

Postprandial state. After ingestion, dietary fat is hydrolyzed and packed in bile acid-containing micelles in the gut and taken up by the enterocytes. FA are incorporated as TAG in chylomicrons that then enter the systemic circulation via the thoracic duct. Chylomicrons contain, in addition to TAG (the major lipid), apolipoprotein(apo) B-48, phospholipids, cholesteryl esters and free cholesterol (DeFronzo et al. 2004). Circulating chylomicrons are hydrolyzed mainly in adipose tissue via the action of LPL, the activity of which is upregulated by insulin postprandially (Sadur and Eckel 1982, Frayn et al. 1994). Both in the fasting and postprandial state, FFA are taken up by adipose tissue through diffusion down a concentration gradient and with the help of fatty acid transport and binding proteins (Stahl 2004).

Remaining circulating chylomicron-remnant particles are removed by the liver (Frayn 2001) majorily via the function of LDL receptors and LDL receptor-related proteins (Cooper 1997). Also the blood flow in adipose tissue increases postprandially (Ruge et al. 2009), and in addition to increased LPL activity, insulin stimulates re-esterification of FA in adipocytes (Frayn et al. 1994, Coleman and Lee 2004) and decreases FFA spillover (Frayn et al. 1995). After ingestion, the net release of FFA in adipose tissue converts to net trapping and there is a rapid fall in total circulating FFA concentration (Bickerton et al. 2007).

Other factors regulating lipid storage and breakdown in adipose tissue. In addition to insulin, lipolysis is regulated by catecholamines that stimulate HSL activity during fasting and under other conditions, such as aerobic exercise (via β_1- and β_2-adrenergic receptors). Other physiological stimulators of lipolysis include TNFα and growth hormone (Coppack et al. 1994, Lafontan and Langin 2009).

During fasting, HSL activity and the rate of lipolysis is enhanced by the action of the protein perilipin located on the surface of TAG droplets (Sztalryd et al. 2003, Tansey et al. 2004, Brasaemle 2007). Both HSL and perilipin action are stimulated by protein kinase A (PKA)-mediated phosphorylation. Under postprandial conditions, perilipin restricts the function of lipases and suppresses TAG breakdown. Positive staining for perilipin has also been used as a marker of viable adipocytes in immunohistochemical sections of both mouse and human adipose tissue (Cinti et al. 2005).

ATGL is another enzyme capable of TAG hydrolysis with high specificity for TAG (Jenkins et al. 2004, Zimmermann et al. 2004). The generated diacylglycerol (DAG) in turn appears to be the main substrate of HSL (Haemmerle et al. 2002, Kraemer and Shen 2002). In addition, the third enzyme in the TAG hydrolytic cascade, monoacylglycerol lipase (MGL), is expressed in adipose tissue and is required to complete lipolysis by hydrolyzing monoacylglycerols into FA and glycerol (Fredrikson et al. 1986).

Another lipase/transacetylase is adiponutrin that is expressed in both adipose tissue and the liver (Wilson et al. 2006). The exact function of adiponutrin is unknown but it has been suggested to participate in both lipolysis and lipogenesis. Its expression is positively associated with obesity (Johansson et al. 2006) and decreased during fasting but increased after re-feeding both in mice and humans (Liu et al. 2004). Recently, genetic variation in adiponutrin gene has been closely associated with liver fat content in humans (discussed in section 4. in *DISCUSSION*).

1.1.2. Glucose metabolism

Adipocytes are highly responsive to insulin and thus in normal subjects even small increases (~5-15 mU/l) in circulating insulin concentration are sufficient to decrease significantly serum FFA concentration (Nurjhan et al. 1986, Bonadonna et al. 1990, Groop et al. 1992). In adipose tissue, insulin stimulates glucose uptake, LPL activity and lipogenesis. Glucose uptake is mediated by facilitated glucose transporter member 4 (GLUT-4) and subsequent

glycolysis is responsible for glycerol-3-phosphate supply for FA re-esterification and TAG synthesis in adipose tissue (Frayn et al. 2006). Adipose tissue accounts for less than 5 % of whole body glucose uptake as determined by early tracer studies (Björntorp et al. 1971, Mårin et al. 1987), and approximately 8 % when measured with a more recent technique using positron emission tomography (PET) and labeled glucose and water during euglycemic hyperinsulinemia (Virtanen et al. 2002).

1.1.3. Adipocytokines

Cytokines are signalling peptides acting in an autocrine, paracrine or endocrine fashion (Cannon 2000). Chemokines are cytokines with chemotactic properties regulating migration of cells (Charo and Ransohoff 2006). Adipocytokines are cytokines secreted from adipose tissue. The definition of adipocytokine varies. Some define them as secretory products exclusively of adipocytes or pre-adipocytes, while others accept also non-fat cells in adipose tissue as a source. These non-fat cells include macrophages, fibroblasts, endothelial cells, lymphocytes and smooth muscle cells and they constitute the stromal vascular fraction (SVF) of adipose tissue (Frayn et al. 2003, Kershaw and Flier 2004). One adipocytokine can originate from several different cell types. In the following text, the term adipocytokine denotes cytokines, chemokines and peptides secreted by any cell type present in adipose tissue.

Adipocytokines constitute a wide spectrum of factors that regulate body weight, insulin sensitivity, glucose and lipid metabolism, and inflammation (Rasouli and Kern 2008). Selected adipocytokines identified in human adipose tissue, their expression and serum concentrations in insulin-resistant states are listed in Table 1. Some adipocytokines play a role in normal physiology. For example, based on studies in genetically engineered mice, adiponectin is an anti-atherogenic adipocytokine that has both insulin-sensitizing and anti-inflammatory effects (discussed in section 2.3. in *REVIEW OF THE LITERATURE*). In humans, lean and insulin-sensitive subjects have higher plasma concentration and adipose tissue expression of adiponectin compared to obese and insulin-resistant subjects. Another example is leptin that is shown to be responsible for normal food intake, energy expenditure and adipose tissue mass (Friedman 2009). Many other adipocytokines are mainly included in inflammation and insulin resistance (discussed in section 2.3. in *REVIEW OF THE LITERATURE*).

Table 1. Selected adipocytokines identified in human adipose tissue, and their gene (G) and/or protein (P) expression in adipose tissue and serum concentration in insulin-resistant subjects*.

Name (abbreviation(s))	*Expression in adipose tissue*	*Serum concentration*	*References*
Acylation stimulating protein (ASP)	↑ (P)	↑	Cianflone et al. 1994, Saleh et al. 1998, Koistinen et al. 2001, Cianflone et al. 2003
Adiponectin (apM1/Acrp30/AdipoQ/GBP28)	↓ (G)	↓	Hu et al. 1996, Arita et al. 1999
Adipsin/Complement factor D	ND	↑	White et al. 1992, Napolitano et al. 1994, Cianflone et al. 2003
Interleukin 1 (IL-1)	↔ (P)	↑	Juge-Aubry et al. 2003, Fain et al. 2004b, Salmenniemi et al. 2004
Interleukin 1 receptor antagonist (IL-1Ra)	↑ (G)	↑	Meier et al. 2002, Juge-Aubry et al. 2003
Interleukin 6 (IL-6)	↑ (G), ↑ (P)	↑	Mohamed-Ali et al. 1997, Fried et al. 1998, Rotter et al. 2003
Interleukin 8 (IL-8)	↑ (G), ↑ (P)	↑	Zozulinska et al. 1999, Bruun et al. 2000, Straczkowski et al. 2002, Rotter et al. 2003
Interleukin 10 (IL-10)	↑ (G)	↑, ↓	Esposito et al. 2003b, Blüher et al. 2005, Juge-Aubry et al. 2005
Interleukin 18 (IL-18)	↑ (G)	↑	Lindegaard et al. 2004, Fischer et al. 2005, Bruun et al. 2007
Leptin	↑ (G)	↑	Zhang et al. 1994, Lönnqvist et al. 1995, Maffei et al. 1995, Klein et al. 1996
Macrophage inflammatory protein 1 α (MIP-1α/CCL3)	↑ (G)	↔	Gerhardt et al. 2001, Huber et al. 2008
Macrophage migration inhibitory factor (MIF)	↑ (G)	↑	Yabunaka et al. 2000, Skurk et al. 2005, Koska et al. 2009
Monocyte chemoattractant protein 1 (MCP-1/CCL2)	↑ (G)	↑, ↔	Gerhardt et al. 2001, Christiansen et al. 2005, Dahlman et al. 2005, Kim et al. 2006
Omentin	↓ (G)	↓	Yang et al. 2006, De Souza Batista et al. 2007
Osteopontin (OPN)	↑ (G)	↑	Gomez-Ambrosi et al. 2007, Kiefer et al. 2008
Plasminogen activator inhibitor 1 (PAI-1)	↑ (G)	↑	Juhan-Vague et al. 1989, Alessi et al. 1997
Resistin (ADSF/FIZZ3)	not expressed in adipocytes	↑, ↔	Savage et al. 2001, Degawa-Yamauchi et al. 2003, Lee et al. 2003b
Retinol binding protein 4 (RBP-4)	↑ (G)	↑	Yang et al. 2005, Janke et al. 2006, Klöting et al. 2007
Tumor necrosis factor α (TNFα)	↑ (G)	↔	Hotamisligil et al. 1995, Kern et al. 1995, Mohamed-Ali et al. 1997
Thrombospondin 1 (TSP-1)	↑ (G)	ND	Ramis et al. 2002, Varma et al. 2008

* Obese vs. lean subjects, *or* equally obese insulin-resistant vs. insulin-sensitive subjects, *or* T2DM vs. control subjects. ↑ indicates increase, ↓ decrease and ↔ no change in gene expression or serum concentration. ND, not determined.

1.2. The liver

1.2.1. Glucose metabolism

After an overnight fast, the liver produces glucose (endogenous glucose production, EGP) at a rate of approximately 2 mg/kg·min in normal subjects (Bondy et al. 1949, DeFronzo et al. 1981, DeFronzo and Ferrannini 1987, Consoli 1992). The liver accounts for most of this via glycogenolysis and gluconeogenesis (GNG) (Ekberg et al. 1999). GNG accounts for ~50 % of hepatic glucose production after an overnight (14 hours) fast and for almost all (~95 %) after a 42-hour fast (Rothman et al. 1991, Landau et al. 1996, Chandramouli et al. 1997).

Hepatic glucose production is suppressed at lower insulin concentration than what is required for stimulation of glucose uptake in skeletal muscle (Rizza et al. 1981, Yki-Järvinen et al. 1987b). These effects on the liver by insulin are mediated directly through both hepatic insulin receptors and downregulation of gluconeogenetic enzymes (Sutherland et al. 1996) and indirectly through effects on pancreatic α-cells, adipose tissue and skeletal muscle (Girard 2006). In addition, studies in mice suggest that inhibition of hepatic glucose production could be partly mediated via neural pathways activated in response to insulin action in the brain (Obici et al. 2002). In normal subjects, complete suppression of hepatic glucose production is achieved at insulin concentration of ~50-60 mU/l and half-maximal suppression at an insulin concentration of ~30 mU/l (Rizza et al. 1981). Hyperglycemia under normoinsulinemic conditions also suppresses hepatic glucose production (DeFronzo et al. 1983). In addition to insulin, glucagon is also an important regulator of hepatic glucose production, particularly in the fasting state. Glucagon counteracts insulin action by stimulating both hepatic GNG (Chiasson et al. 1975) and glycogenolysis (Magnusson et al. 1995). In addition to glucagon, other insulin counterregulatory hormones include cortisol, adrenalin and noradrenalin.

After ingestion of oral glucose or a mixed meal, increases in insulin and glucose concentrations, and a decrease in glucagon concentration, suppress hepatic glucose production almost completely (Taylor et al. 1996, Singhal et al. 2002). Concerning disposition of an oral glucose load (1 g/kg body weight), splanchnic tissues take up approximately 30 % during 5 hours (Kelley et al. 1988). Hyperglycemia stimulates splanchnic glucose uptake also independent of insulin concentration (DeFronzo et al. 1983).

1.2.2. Lipid metabolism

Origin of intrahepatic TAG. The uptake of FFA and secretion of lipids in the form of VLDL by the liver also depends on the nutritional state (Frayn et al. 2006). In the fasting state, the majority of circulating FFA that are taken up by the liver, originate from adipose tissue lipolysis and these FFA are also the main source (~80 %) of FA incorporated in VLDL. In the fasting state less than 5 % of intrahepatocellular TAG in normal subjects originates from hepatic *de novo* lipogenesis (DNL) (Barrows and Parks 2006). Other sources of intrahepatocellular TAG during fasting include FA delivered to the liver in the form of

VLDL-remnants and spillover of FFA from intravascular lipolysis in peripheral tissues (Donnelly et al. 2005, Goldberg and Ginsberg 2006).

In the postprandial state, adipose tissue lipolysis is suppressed by insulin but still accounts for the majority (~45 %) of FA used for hepatic VLDL synthesis while DNL accounts only for a small fraction (~8 %) of the FA incorporated in intrahepatic TAG (Barrows and Parks 2006). Uptake of chylomicron remnants and the spillover pathway also account for a small part (~5-15 %) of VLDL-TAG assembly (Barrows et al. 2005). Lipid stores of the liver are dynamic, since ^{13}C-labeled FA in a lipid mixture ingested along with normal breakfast, showed peak incorporation in hepatic TAG stores after 6 hours when measured with ^{13}C-MRS (magnetic resonance spectroscopy) in normal subjects (Ravikumar et al. 2005). Labeled FA were also rapidly replaced by non-labeled FA in the subsequent mixed meal.

Fate of FA in the liver. In the liver, FA can be directed in oxidation, ketone body formation, TAG storage or phospholipid synthesis, or they can be incorporated into VLDL. Under fasting conditions, low insulin and high glucagon concentrations favor mitochondrial FA oxidation and VLDL synthesis over storage (Frayn et al. 2006, Tessari et al. 2009). On the other hand, dietary FA are rapidly incorporated into the liver TAG stores (Ravikumar et al. 2005) instead of VLDL synthesis (Gibbons et al. 2004) in the postprandial state. In normal subjects, insulin decreases apoB-100 and VLDL-TAG production under hyperinsulinemic normoglycemic conditions (Malmström et al. 1997, Julius 2003). This results from direct suppression of apoB-100 synthesis and VLDL assembly by insulin and from suppression of hepatic uptake of FFA via the antilipolytic effect of insulin (Havel et al. 1970, Lewis et al. 1995, Ginsberg et al. 2005).

1.3. Skeletal muscle

1.3.1. Glucose metabolism

After an overnight fast, whole body glucose uptake averages approximately 2 mg/kg·min and is mostly insulin-independent. The brain takes up approximately 50% of this glucose, while skeletal muscle accounts for only approximately 10 % of whole body glucose uptake in the fasting state (Yki-Järvinen 1993). However, skeletal muscle is the major tissue for insulin-stimulated glucose extraction *in vivo* (DeFronzo et al. 1985), and under intravenously maintained hyperinsulinemic normoglycemic conditions, glucose replaces FFA as the main fuel for skeletal muscle (Kelley et al. 1988, Kelley et al. 1990). Muscle glucose uptake increases 8-fold under hyperinsulinemia (DeFronzo et al. 1981, Yki-Järvinen et al. 1987b, Nuutila et al. 1992) and after ingestion of a normal mixed meal (Ruge et al. 2009). Of an oral glucose load (1 g/kg body weight) or after a mixed meal containing ~80 g glucose, skeletal muscle takes up approximately 30 % of the glucose during 5- to 6-hour period. Most glucose (~50 %) taken up by skeletal muscle is oxidized for energy, while the rest is stored as glycogen and glycolyzed into lactate, pyruvate and alanine (Kelley et al. 1988, Woerle et al. 2003). The maximal effective concentration of insulin to stimulate glucose uptake in skeletal muscle in normal insulin-sensitive subjects is ~200 mU/l, while the half-maximal

concentration is ~80 mU/l (Yki-Järvinen et al. 1987a). However, in everyday life in normal insulin-sensitive subjects the circulating concentration of insulin rarely exceeds 50 mU/l.

Concerning the mechanisms of insulin-stimulated glucose uptake in muscle (and in adipose tissue), insulin first binds to the insulin receptor and induces its autophosphorylation (Kasuga et al. 1982). This leads to several intra-cellular phosphorylation-dephosphorylation cascades (White 2003) including insulin-receptor substrate proteins 1 and 2 (IRS-1/2), phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B (PKB) that are the most important links between insulin receptor binding and intracellular effects of insulin (Saltiel and Kahn 2001). These cascades lead to the translocation and fusion of the insulin-sensitive GLUT-4 from intracellular vesicles to the cell membrane (Klip and Paquet 1990, Guma et al. 1995) that launches the anabolic effects in glucose, lipid and protein metabolism. Insulin also regulates the intracellular enzyme hexokinase (Mandarino et al. 1995) that phosphorylates transported glucose into glucose-6-phosphate (G6P), which is used for glycogen synthesis and glycolysis.

1.3.2. Lipid metabolism

In the fasting state, circulating FFA are the major oxidative substrates for skeletal muscle (Andres et al. 1956, Baltzan et al. 1962). FFA originate from plasma albumin-bound FFA or from LPL-catalyzed intravascular VLDL-TAG lipolysis in adipose tissue. There is almost no extraction of VLDL-TAGs in muscle, while postprandially the extraction of FA from chylomicrons by LPL is quite efficient. Nevertheless, most of dietary FA from chylomicrons are diverted into adipose tissue. FA can be stored as TAG in adipocytes between muscle fibers or inside myocytes as intramyocellular lipid (IMCL) (Frayn et al. 2006). Only approximately 5 % of postprandial whole body TAG storage occurs in muscle tissue (Ravikumar et al. 2005). IMCL content is inversely related to insulin sensitivity in normal and obese sedentary subjects (Krssak et al. 1999, Boden et al. 2001). However, IMCL is paradoxically increased and positively associated with insulin sensitivity in aerobic endurance-trained athletes for reasons, which are incompletely understood (Thamer et al. 2003).

Exercise affects muscle fuel selection (Henriksson 1995). At rest, FFA are the dominant energy source and only a minor part of muscle fuel derives from plasma glucose. During prolonged low to moderate intensity exercise FFA (from both circulation and IMCL) still play an important role as energy source, but during initial period and with high-intensity exercise the use of muscle glycogen stores and plasma-derived glucose dominate as fuel (Ahlborg et al. 1974).

2. Insulin resistance in adipose tissue

Growing evidence links low-grade inflammation in adipose tissue with obesity and insulin resistance. This inflammation is characterized by increased infiltration of macrophages and increased expression of pro-inflammatory adipocytokines in adipose tissue (Rasouli and Kern 2008). However, the sequence of events leading to inflammation and the mechanisms that link adipose tissue inflammation to insulin resistance are still poorly understood.

2.1. Defects in *in vivo* insulin action in adipose tissue

Insulin resistance and fasting serum insulin concentration tend to increase with increasing obesity, but not all obese subjects are insulin-resistant (Bogardus et al. 1985, Abate et al. 1995). In adipose tissue, insulin is responsible for increasing glucose uptake, LPL activity and lipogenesis and, on the other hand, for decreasing HSL activity and adipocyte TAG lipolysis. The classic hallmark of insulin resistance in adipose tissue is the defective antilipolytic effect of insulin that leads to increased circulating FFA concentration (Coppack et al. 1994).

Adipose tissue FFA release and circulating FFA concentration, under both fasting and postprandial conditions, are increased in obese and insulin-resistant compared to normal weight and insulin-sensitive subjects (Opie and Walfish 1963, Baldeweg et al. 2000). Lipolysis and the release of FFA by adipose tissue measured by labeled $^{2}H_{5}$-glycerol and ^{14}C-palmitate respectively, are suppressed by insulin in both lean and obese subjects in a dose-dependent manner under euglycemic hyperinsulinemic conditions (Campbell et al. 1994). However, in obese subjects, insulin doses that decrease FFA release by adipose tissue are significantly higher compared to lean subjects. Several studies using euglycemic hyperinsulinemia have also shown that the suppression of FFA release by insulin is defective in obese and insulin-resistant subjects (Yki-Järvinen and Taskinen 1988, Jensen et al. 1989, Groop et al. 1992, Virtanen et al. 2005). Some studies, however, found no statistically significant difference in the antilipolytic effects of insulin between obese and lean subjects under hyperinsulinemic conditions (Howard et al. 1984, Zuniga-Guajardo et al. 1986).

In everyday life, insulin resistance of adipose tissue lipolysis is especially important under postprandial conditions (Frayn 2001). During fasting, obese subjects show decreased clearance of VLDL-TAG in adipose tissue compared to lean subjects (Potts et al. 1995). After a standard mixed meal, the normal stimulation of LPL and suppression of HSL activity are blunted in obese compared to lean subjects, and adipose tissue continues to release FFA in obese but not in lean subjects (Coppack et al. 1992, Frayn et al. 1996). In addition, after a mixed meal, adipose tissue extraction of FFA from TAG-rich chylomicrons and VLDL is decreased in obese compared to lean subjects (Potts et al. 1995). Glucose uptake in adipose tissue is also resistant to insulin action in obese and insulin-resistant subjects compared to non-obese counterparts (Virtanen et al. 2001). The quantitative contribution of adipose tissue to whole body glucose uptake, however, is small (less than ~5 %) even in the obese subjects (Björntorp et al. 1971, Mårin et al. 1987, Virtanen et al. 2002) and thus unlikely contributes to decrease in glucose disposal in insulin resistance.

Although the visceral (i.e. intra-abdominal) fat depot constitutes ~10-20 % of the total body fat mass in humans (Ross et al. 1993, Abate et al. 1995), it is suggested to be more harmful compared to subcutaneous fat concerning the risk of insulin resistance, T2DM and cardiovascular diseases (CVD) (Montague and O'Rahilly 2000, Mathieu et al. 2009). Visceral fat is considered metabolically more active than subcutaneous fat in both lipid and glucose metabolism, and in unfavorable cytokine secretion (Després and Lemieux 2006). Lipolysis and FFA release and insulin-stimulated glucose uptake are increased in visceral compared to subcutaneous adipose tissue (Arner 1995, Lafontan and Berlan 2003, Nielsen et al. 2004, Virtanen et al. 2005). In both normal weight and morbidly obese subjects, the number of macrophages, measured by immunohistochemical staining, is increased in visceral compared to subcutaneous adipose tissue (Bornstein et al. 2000, Cancello et al. 2006). In addition to body mass index (BMI) that represents general adiposity, waist circumference and waist-to-hip ratio (W/H) are strongly associated with overall mortality in a large prospective study (Pischon et al. 2008). Waist circumference represents mainly visceral and upper-body obesity. Upper-body subcutaneous fat depot is the major source of FFA release in whole body (Koutsari and Jensen 2006), although the FFA released by visceral adipose tissue possess a direct route to the liver via the portal vein (Björntorp 1990).

2.2. Inflammation

Experimental animal models have proposed strong evidence of the important role of inflammatory pathways in the pathophysiology of insulin resistance (Hotamisligil et al. 1993, Yuan et al. 2001, Arkan et al. 2005). Causality is difficult to prove in human studies but there is evidence of a relationship between inflammation and insulin resistance. For example, the expression of pro-inflammatory cytokines, such as TNFα, and tissue infiltration of inflammatory cells, are increased in adipose tissue of obese subjects (De Luca and Olefsky 2008).

In 2003, two groups reported that obesity and insulin resistance are associated with increased macrophage accumulation and chronic low-grade inflammation in adipose tissue in mice and humans (Weisberg et al. 2003, Xu et al. 2003b). Macrophages are suggested to be major contributors to the inflammatory changes observed in adipose tissue. Expression of macrophage marker gene Cluster of differentiation 68 (CD68) is significantly higher in adipose tissue of obese when compared to lean subjects. Adipocyte cell size is closely correlated to BMI and insulin resistance and also to macrophage infiltration (Hirsch and Batchelor 1976, Coppack 2001, Weisberg et al. 2003). Transplantation studies suggested that the adipose tissue macrophages are bone marrow-derived monocytes infiltrating the site of inflammation (Weisberg et al. 2003) rather than preadipocytes differentiating into macrophage-like cells (Charriere et al. 2003). Other studies have confirmed the increased infiltration of adipose tissue macrophages in obese or insulin-resistant subjects by assessing the gene expression or immunohistochemical staining of macrophage markers (Cinti et al. 2005, Di Gregorio et al. 2005, Pietiläinen et al. 2006, Coenen et al. 2007). In contrast to inflamed adipose tissue, human skeletal muscle shows almost no macrophage infiltration in obese insulin-resistant subjects (Bruun et al. 2006). The relationships between adipose tissue

inflammation and macrophage infiltration with liver fat content, and with whole body insulin sensitivity have not been previously determined in humans *in vivo*.

In addition to CD68 (Holness and Simmons 1993, Weisberg et al. 2003), also other gene markers for macrophage tissue infiltration have been used. A disintegrin and metallopeptidase domain 8 (ADAM8) (Xu et al. 2003b) and epidermal growth factor module-containing mucin-like hormone receptor 1 (EMR1) (McKnight and Gordon 1998) are monocyte- and macrophage-specific proteins, and integrin α M (ITGAM) is a leukocyte surface adhesion molecule found in monocytes, macrophages, neutrophils and NK cells (Solovjov et al. 2005). All these genes show absent or only minor expression in human adipocytes (Khazen et al. 2005, Lee et al. 2005).

The mechanisms underlying macrophage infiltration in adipose tissue remain unresolved. It has been suggested that the increase in adipocyte cell size could promote adipocyte cell dysfunction, cell death and necrosis (Cinti et al. 2005). Expansion of adipose tissue may lead to hypoxia and activation of the intracellular inflammatory pathways leading to increased expression and secretion of inflammatory and chemoattractant adipocytokines (Wang et al. 2007, Pasarica et al. 2009). Endoplasmic reticulum (ER) stress can also activate inflammatory pathways and adipocytokine expression (Ozcan et al. 2004, Zhang and Kaufman 2008) and is associated with initiation of inflammation and insulin resistance (Schenk et al. 2008). ER stress and dysfunction can be triggered by hypoxia and chronic overflow of FFA that also characterize obesity (Gregor and Hotamisligil 2007, Boden et al. 2008).

In both obese mice and men, using immunohistochemistry and electron microscopy, macrophages have been shown to form crown-like structures (CLS) consisting of several macrophages surrounding necrotic, perilipin-negative, adipocytes (Cinti et al. 2005). These structures scavenge the cell debris, and at the same time actively secrete cytokines and recruit more macrophages, thereby maintaining the inflammatory reaction in adipose tissue. Adipocyte cell size strongly predicts the number of macrophages and CLS in adipose tissue, even independent of total fat mass (Cinti et al. 2005). In human adipocytes *in vitro*, large hypertrophic cells presented a proinflammatory cytokine secretion profile compared to smaller cells (Skurk et al. 2007). In morbidly obese subjects, histologically determined macrophage number correlated positively with adipocyte cell size in both visceral and subcutaneous adipose tissue (Cancello et al. 2006). Thus, it has been suggested that in obesity adipocytes reach a critical cell size after which the function of the cell and whole tissue changes into an inflammatory direction (Cinti 2009). This is characterized by increased expression of inflammatory adipocytokines, infiltration of macrophages, as well as impaired adipogenesis, lipid storage and accumulation of ectopic fat (e.g. to the liver) (Figure 1).

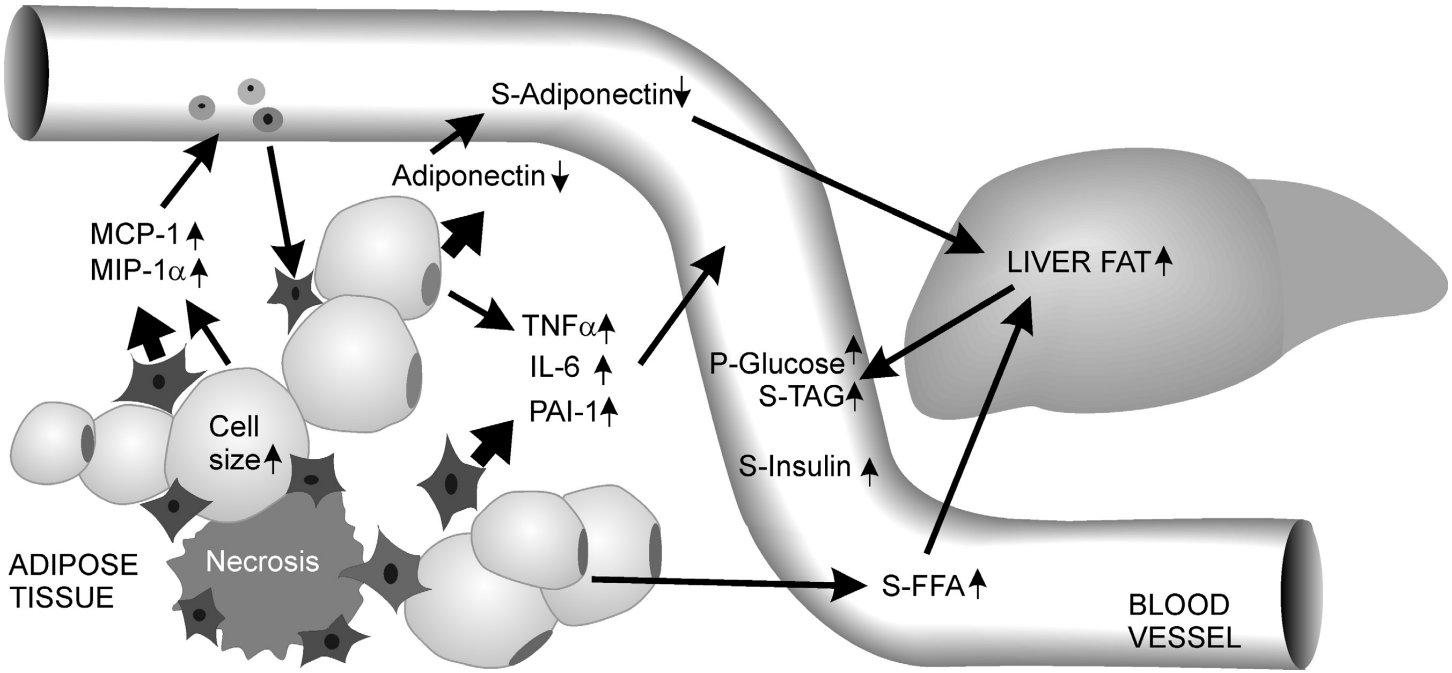

Figure 1. Adipose tissue inflammation in obesity and its relationships to circulating factors and liver fat content. Light-grey and round cells are adipocytes. The medium-grey cell with irregular membrane is necrotic adipocyte surrounded by numerous macrophages (dark-grey star-shaped cells). Small round cells in blood vessel are circulating monocytes recruited to inflamed adipose tissue and differentiated into macrophages. Thick arrows originating from adipocytes and/or macrophages indicate major contribution to adipocytokine secretion and thin arrows minor contribution. For references, see text in sections 2.1., 2.2. and 2.3. in *REVIEW OF THE LITERATURE*.

Concerning the mechanisms by which inflammation in adipose tissue could be linked to insulin resistance, inhibitor of IκB (IKK-β) and C-Jun amino-terminal kinase (JNK) are suggested to play a role (Schenk et al. 2008). IKK-β is an important coordinator of cellular inflammatory responses via inhibiting IκB that, in turn, inhibits activation of nuclear factor κB (NFκB), an inflammatory transcription factor. JNK is another important intracellular modulator of inflammatory pathways, also associated with insulin resistance (Hirosumi et al. 2002). Both IKK-β and JNK are serine kinases capable of inhibiting the function of IRS-1 and thereby insulin signaling. Interestingly, in knock-out (KO) studies where IKK-β or JNK were depleted specifically from myeloid cells (monocytes, macrophages, neutrophils, lymphocytes) but not from adipose tissue, the liver or skeletal muscle, mice were protected from whole body insulin resistance (Arkan et al. 2005, Solinas et al. 2007). In intervention studies using salicylates, inhibitors of IKK-β, insulin resistance was ameliorated in both rodents and humans (Kim et al. 2001, Hundal et al. 2002). TNFα-deficient myeloid cells also show enhanced insulin sensitivity (De Taeye et al. 2007).

Circulating monocytes and macrophages can be divided into two major subsets with heterogenic properties (Gordon and Taylor 2005). Proinflammatory, or classically activated, M1-macrophages show increased reactivity to lipopolysaccharide (LPS), whereas anti-inflammatory, or alternatively activated, M2-macrophages are suggested to associate with normal adipocyte function and insulin sensitivity, and to show increased IL-10 expression (Lumeng et al. 2007). In obese mice and humans, M1/M2 ratio in adipose tissue is increased and with high-fat feeding in mice, the phenotype of macrophages changes from M2 into M1 contributing to insulin resistance (Lumeng et al. 2007, Aron-Wisnewsky et al. 2009). The proinflammatory switch may originate from bone marrow-derived monocytes that are shown to be in proinflammatory state in obese subjects (Ghanim et al. 2004) or by changes in adipose tissue microenvironment that favors inflammation (Stout et al. 2005). Also different growth factors (granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF)) may determine the phenotype and inflammatory properties of differentiated macrophages (Waldo et al. 2008). In humans, weight loss after bariatric surgery decreased M1/M2 ratio in subcutaneous adipose tissue (Aron-Wisnewsky et al. 2009). LPS-stimulated inflammatory gene expression responses in human monocyte-derived macrophages and possible differences between insulin-resistant and sensitive subjects have not been previously studied.

2.3. Adipocytokines and insulin resistance

Monocyte chemoattractant protein 1

MCP-1 (also known as CCL2) is a mononuclear cell-specific chemokine and the C-C motif chemokine receptor 2 (CCR2) is its specific receptor. MCP-1 is expressed in many cell types including adipocytes but other cells in adipose tissue, especially macrophages, have been proposed to be its predominant source (Murao et al. 1999, Bruun et al. 2005, Fain and Madan 2005). MCP-1 and CCR2 have been suggested to be crucial in macrophage recruitment, adipose tissue inflammation and insulin resistance (Neels and Olefsky 2006).

In ob/ob mice, expression of MCP-1 in the liver is less than 10 % of that in adipose tissue (Sartipy and Loskutoff 2003). MCP-1 induces insulin resistance and decreases expression of GLUT-4, LPL and nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) in adipocytes *in vitro*. In genetically obese db/db-mice or in wild-type (WT) mice with high-fat diet-induced obesity, the adipose tissue expression and serum concentration of MCP-1 are increased compared to controls (Kanda et al. 2006). Overexpression of MCP-1 selectively in adipose tissue in mice markedly increases serum MCP-1 concentration, macrophage infiltration in adipose tissue, insulin resistance and hepatic fat accumulation. Obese MCP-1-KO mice, on the other hand, are protected from these changes when compared to equally obese WT mice, according to some (Kanda et al. 2006) but not all (Inouye et al. 2007, Kirk et al. 2008) studies. Overexpression of MCP-1 in adipose tissue also increases adipose tissue expression of TNFα and IL-6, and plasma concentration of FFA (Kamei et al. 2006). CCR2-KO mice have also been suggested to exhibit reduced macrophage infiltration and inflammatory responses, and increased adiponectin expression in adipose tissue. These mice show decreased insulin resistance and hepatic steatosis (Weisberg et al. 2006). Similar results were not found, however, in a previous study (Chen et al. 2005a).

In obese humans, expression of MCP-1 and CCR2 are increased in both subcutaneous and visceral adipose tissue compared to lean subjects (Christiansen et al. 2005, Dahlman et al. 2005, Huber et al. 2008). Gene expression of MCP-1 is also associated with that of macrophage marker CD68 in both adipose tissue depots in humans. MCP-1 protein secretion by cultured human adipose tissue is increased in obesity and is higher in visceral than in subcutaneous adipose tissue (Bruun et al. 2005, Dahlman et al. 2005). Data of circulating concentration of MCP-1 have been variable. Some studies found no difference in serum MCP-1 concentration between obese and lean subjects or in venous blood draining subcutaneous adipose tissue (Dahlman et al. 2005), while others found increased serum MCP-1 concentration in obese compared to lean subjects, and also a correlation between adipocyte MCP-1 expression, serum MCP-1 concentration and BMI (Christiansen et al. 2005, Kim et al. 2006).

Concerning regulation of MCP-1 in humans, weight loss induced by hypocaloric diet (Bruun et al. 2006) or by bariatric surgery (Cancello et al. 2005) decreases macrophage infiltration and MCP-1 gene expression in subcutaneous adipose tissue. Pioglitazone treatment for 10 weeks decreases macrophage number and expression of CD68 and MCP-1 in subcutaneous adipose tissue, while metformin had no such effects (Di Gregorio et al. 2005). In human adipose tissue *in vitro*, TNFα, IL-1β and IL-6 increase MCP-1 secretion (Bruun et al. 2005). Data of regulation of MCP-1 by insulin are inconsistent. In humans under euglycemic hyperinsulinemic conditions, adipose tissue gene expression of MCP-1 has been reported to increase only in lean subjects (Murdolo et al. 2007). In the latter study, neither obese nor lean subjects showed change in serum MCP-1 concentration. Adipose tissue interstitial MCP-1 concentration, on the other hand, is increased in both obese and lean subjects under hyperinsulinemic conditions (Murdolo et al. 2007, Siklova-Vitkova et al. 2009). The acute

effects of insulin *in vivo* on adipose tissue expression and serum concentration of MCP-1 have not been compared in insulin-sensitive and insulin-resistant subjects.

Macrophage inflammatory protein 1α

Macrophage inflammatory protein 1α (MIP-1α, also known as CCL3) is secreted by preadipocytes, mature adipocytes and activated macrophages and has chemotactic and proinflammatory effects (Gerhardt et al. 2001, Maurer and von Stebut 2004). Macrophages from human subcutaneous and visceral adipose tissue show higher expression of MIP-1α than isolated adipocytes (Curat et al. 2006). LPS, TNFα and IL-1 stimulate MIP-1α production while IL-10 and dexamethasone have the opposite effect (Maurer and von Stebut 2004). Macrophage-conditioned medium increases MIP-1α secretion by human visceral adipocytes *in vitro* (Bassols et al. 2009). Studies on MIP-1α in humans *in vivo* are scarce and gene expression in adipose tissue in insulin-resistant and sensitive subjects is unknown.

Tumor necrosis factor α

TNFα is an important and extensively studied proinflammatory cytokine capable of inducing and mediating insulin resistance in different cell lines and animal models (Hotamisligil et al. 1993, Hotamisligil et al. 1994, Liu et al. 1998). LPS-stimulated TNFα production is more abundant by whole human adipose tissue than by isolated adipocytes (Sewter et al. 1999). Macrophages are suggested as the predominant source of TNFα in adipose tissue (Weisberg et al. 2003, Fain et al. 2004a, Di Gregorio et al. 2005). However, it is unclear whether merely the number, or also the location and properties of TNFα-secreting macrophages, differ in obesity and insulin resistance.

Adipose tissue expression of TNFα is increased in obese and insulin-resistant mice and associates positively with adiposity (Hotamisligil et al. 1993). TNFα decreases IRS-1 and GLUT-4 expression in 3T3-L1 adipocytes and thereby inhibits insulin-stimulated glucose uptake (Peraldi et al. 1997). TNFα also inhibits expression of PPARγ that is important in adipocyte differentiation and the target for anti-diabetic agents, thiazolidinediones (also known as glitazones). TNFα decreases LPL activity and stimulates intracellular lipolysis in adipocytes (Zhang et al. 2002). In obese mice, TNFα-KO protects from insulin resistance, decreases circulating FFA concentration and improves insulin signaling in muscle and adipose tissue (Uysal et al. 1997).

In human adipocytes, treatment with TNFα decreases IRS-1 and PI3K activity with a simultaneous decrease in glucose uptake (Liu et al. 1998) and decreases the expression of adiponectin (Bruun et al. 2003). Adipose tissue gene expression of TNFα is increased in obese compared to lean subjects (Hotamisligil et al. 1995, Kern et al. 1995, Pietiläinen et al. 2006). Adipose tissue expression and serum concentration of TNFα correlate with liver-derived acute phase protein C-reactive protein (CRP) in obese subjects (Maachi et al. 2004). Recently in a large cohort study, serum TNFα concentration correlated significantly with homeostasis model insulin resistance (Hivert et al. 2008). On the other hand, some studies found no

correlation between adipose tissue TNFα expression and obesity or insulin sensitivity (Frittitta et al. 1997, Montague et al. 1998, Koistinen et al. 2000). In obese T2DM patients, 4-week treatment with recombinant-engineered human TNFα-neutralizing antibody did not affect whole body insulin sensitivity (Ofei et al. 1996) while this was effective previously in mice (Hotamisligil et al. 1993). A subsequent study, however, suggested that chronic, instead of acute, anti-TNFα treatment could improve insulin sensitivity in humans (Yazdani-Biuki et al. 2004). Additionally, the endocrine effects of TNFα on systemic insulin resistance in humans have been questioned by adipose tissue arteriovenous difference studies that show no significant secretion of TNFα by subcutaneous adipose tissue (Mohamed-Ali et al. 1997). This suggests primarily autocrine/paracrine function for TNFα in human adipose tissue.

Human adipose tissue TNFα expression decreases markedly after weight reduction (Hotamisligil et al. 1995, Kern et al. 1995, Bruun et al. 2006) while acquired obesity increases adipose tissue TNFα and CD68 expression (Pietiläinen et al. 2006)., The results of acute regulation of adipose tissue expression of TNFα by insulin in humans are not completely coherent. Some studies show no effect of insulin on TNFα expression *in vitro* (Sewter et al. 1999) while others represent increased expression in healthy subjects *in vivo* (Krogh-Madsen et al. 2004). Interestingly, in human monocyte cell line and monocyte-derived human macrophages *in vitro*, insulin has been shown to stimulate expression of TNFα more than any other gene (Iida et al. 2001). Differences in adipose tissue TNFα expression in non-diabetic insulin-sensitive and resistant subjects and acute effects of hyperinsulinemia on TNFα expression have not been previously assessed.

Interleukin 6

IL-6 is a cytokine of the immune system and the main cytokine involved in acute phase reaction and associated with insulin resistance (Feve and Bastard 2009). In humans, significant amounts of IL-6 are secreted by adipose tissue into the circulation, in contrast to TNFα (Mohamed-Ali et al. 1997). Intra-abdominal adipose tissue seems to secrete more IL-6 than subcutaneous depot and adipocytes have been suggested to account for only ~10 % of total IL-6 secretion in adipose tissue, and thus, the SVF (mainly macrophages) primaly contributes to IL-6 production (Fried et al. 1998, Weisberg et al. 2003, Fain et al. 2004b).

In vitro, IL-6 decreases IRS-1, GLUT-4 and adiponectin expression in 3T3-L1 adipocytes (Fasshauer et al. 2003b, Rotter et al. 2003). Mice with IL-6-KO unexpectedly showed increased whole body fat mass, impaired glucose tolerance (IGT), dyslipidemia and leptin insensitivity (Wallenius et al. 2002). However, these results were not reproduced in a later study with a similar design (Di Gregorio et al. 2004). Overexpression of IL-6 selectively in skeletal muscle in mice, results in decreased body weight, but also in hyperinsulinemia, impaired glucose uptake in muscle, and inflammatory changes in the liver (Franckhauser et al. 2008).

In humans, circulating IL-6 concentration and adipose tissue IL-6 protein content and secretion are positively associated with obesity and insulin resistance (Mohamed-Ali et al.

1997, Vozarova et al. 2001, Bastard et al. 2002, Maachi et al. 2004). Adipose tissue gene expression of IL-6 is correlated to adipocyte cell size (Sopasakis et al. 2004), and is increased in insulin-resistant compared to sensitive subjects, independent of body weight (Rotter et al. 2003). Circulating IL-6 is also associated with serum FFA concentration (Bastard et al. 2002) and IL-6 have been shown to stimulate lipolysis *in vivo* in healthy subjects (Lyngso et al. 2002). IL-6 downregulates adiponectin and PPARγ gene expression in adipose tissue *in vitro*. Interestingly, interstitial concentration of IL-6 in human subcutaneous adipose tissue appears ~100 times higher than that in serum (Sopasakis et al. 2004), and human adipocytes also express IL-6 receptors (Bastard et al. 2002). These data suggest both endocrine and auto or paracrine effects for this adipocytokine. IL-6 is also the main stimulator of hepatic acute phase protein production (Gabay and Kushner 1999). Thus, serum concentration of IL-6 and concomitant IL-6-dependent CRP, amyloid A and fibrinogen concentrations are increased in subjects with IGT and T2DM compared to healthy control subjects (Müller et al. 2002).

Some studies suggest no relationship between circulating IL-6 and deteriorated insulin-stimulated glucose disposal and propose even insulin-sensitizing effects for IL-6 (Carey et al. 2006). In skeletal muscle during acute exercise, production of IL-6 increases and circulating IL-6 concentration increases 100-fold (Febbraio and Pedersen 2002). In muscle, the present evidence suggests, that IL-6 may increase rather than decrease insulin sensitivity (Glund et al. 2007).

Glucocorticoids inhibit and catecholamines and TNFα stimulate expression of IL-6 in adipose tissue (Fried et al. 1998, Vicennati et al. 2002, Fasshauer et al. 2003a). IL-6 secretion is increased dose-dependently by insulin in adipocytes *in vitro* (Vicennati et al. 2002). Under euglycemic hyperinsulinemic conditions in humans, both subcutaneous adipose tissue gene expression and plasma concentration of IL-6 increase (Krogh-Madsen et al. 2004). In the latter study, no change in human skeletal muscle IL-6 expression was observed. In another study under hyperinsulinemic conditions, simultaneous infusion of human recombinant IL-6 increased, rather than decreased, whole body glucose disposal with no effect on hepatic glucose production (Carey et al. 2006). Weight loss induced by hypocaloric diet in humans decreases both serum concentration and adipose tissue gene expression of IL-6 (Bastard et al. 2000).

Thus, despite strong relationship with inflammation, the contribution of IL-6 to insulin resistance is under vivid debate and more *in vivo* studies in humans are warranted.

Adiponectin

Adiponectin was identified by four research groups in the mid-1990s (Scherer et al. 1995, Hu et al. 1996, Maeda et al. 1996, Nakano et al. 1996) and was shown to be exclusively produced by mature adipocytes. Two adiponectin receptors, AdipoR1 and AdipoR2, mediating the effects of adiponectin have been characterized in the liver, skeletal muscle (Yamauchi et al. 2003) and in adipose tissue (Rasmussen et al. 2006).

Adiponectin has been suggested to function as an insulin-sensitizing and anti-inflammatory adipocytokine (Kadowaki et al. 2006). In human hepatic cell line *in vitro*, biosynthesis and secretion of anti-atherogenic high-density lipoprotein (HDL) are increased by adiponectin (Matsuura et al. 2007). Mice deficient of adiponectin gene expression show notable insulin resistance and atherosclerosis *in vivo* (Kubota et al. 2002) and are characterized by increase in both adipose tissue expression and circulating concentration of TNFα (Maeda et al. 2002). Lipoatrophic mice deficient of subcutaneous adipose tissue show absent circulating adiponectin and insulin resistance, and infusion of adiponectin in these mice decreases insulin resistance (Yamauchi et al. 2001). Overexpression of adiponectin prevents diabetic phenotype of obese ob/ob mice (Kim et al. 2007). In addition, macrophage infiltration and TNFα expression in adipose tissue, circulating IL-6 concentration and liver fat content are decreased in these mice overexpressing adiponectin. Injections of adiponectin in mice decrease body weight and circulating FFA, glucose and insulin concentrations (Fruebis et al. 2001), and decrease glucose production by the liver (Combs et al. 2001). Adiponectin infusion also decreases liver fat content in both lipoatrophic (Yamauchi et al. 2001) and obese mice (Xu et al. 2003a).

In human endothelial cells *in vitro*, beneficial cellular effects of adiponectin are suggested to involve inhibition of TNFα-mediated activation of NFκB (Ouchi et al. 2000). Also in human adipose tissue SVF cells and in monocyte-derived human macrophages *in vitro*, adiponectin incubation has been suggested to increase the anti-inflammatory M2-phenotype over proinflammatory M1-phenotype (Ohashi et al. 2009). In humans *in vivo*, adiponectin gene expression in adipose tissue is decreased in obese compared to lean subjects (Hu et al. 1996, Bruun et al. 2003). Also circulating concentration of adiponectin decreases in obesity (Arita et al. 1999), T2DM (Weyer et al. 2001) and in the MetS (Trujillo and Scherer 2005). Circulating adiponectin also correlates negatively with serum insulin concentration and positively with insulin sensitivity, as measured using the euglycemic hyperinsulinemic clamp technique (Weyer et al. 2001). Plasma adiponectin concentration correlates inversely with hepatic glucose production (Bajaj et al. 2004) and with expression and secretion of TNFα and IL-6 in adipose tissue (Bruun et al. 2003, Kern et al. 2003). A recent meta-analysis suggests a correlation between a higher circulating adiponectin concentration and a lower risk of T2DM in different ethnic groups independent of gender and BMI (Li et al. 2009).

Concerning regulation of adiponectin in humans, treatment with TNFα and IL-6 with its soluble receptor (IL-6sR) decrease expression of adiponectin in adipose tissue *in vitro* (Bruun et al. 2003). Treatment with glitazones increases both plasma and adipose tissue expression of adiponectin in normal, obese and T2DM subjects without changes in body weight (Maeda et al. 2001, Yu et al. 2002b, Tiikkainen et al. 2004). Glitazone-induced decrease in liver fat content inversely correlates with concomitant increase in plasma adiponectin in T2DM patients (Tiikkainen et al. 2004). Weight loss in obese non-diabetic subjects increase plasma adiponectin concentration and this increase correlates with enhanced insulin sensitivity (Yang et al. 2001, Bruun et al. 2003, Esposito et al. 2003a).

Insulin stimulates adiponectin secretion in visceral but not in subcutaneous human adipocytes *in vitro* (Motoshima et al. 2002). *In vivo* studies in humans show no effect of up to 3 hours of euglycemic hyperinsulinemia on subcutaneous adipose tissue expression of adiponectin in healthy insulin-resistant (Lihn et al. 2003) or T2DM subjects (Koistinen et al. 2004). Longer, 5-hour euglycemic hyperinsulinemia, however, was suggested to decrease plasma adiponectin in lean, obese and T2DM subjects, with no differences between the groups (Yu et al. 2002b). This discrepancy warrants further *in vivo* studies in humans to clarify the acute effects of insulin on adipose tissue expression of adiponectin.

Plasminogen activator inhibitor 1

Plasminogen activator inhibitor 1 (PAI-1) is the physiological rapid acting inhibitor of fibrinolysis and involved in the atherothrombotic process (Skurk and Hauner 2004). Adipose tissue is an important site of expression and secretion of PAI-1 in humans (Alessi et al. 1997). Other PAI-1 producing tissues include the liver, endothelium and thrombocytes. In adipose tissue, SVF has been suggested to be the predominant cell fraction of PAI-1 secretion (Bastelica et al. 2002).

Mice deficient of PAI-1 expression show resistance to high-fat diet-induced obesity, have increased insulin sensitivity and adipose tissue PPARγ and adiponectin expression, and decreased muscle and hepatic lipid content compared to WT mice (Ma et al. 2004). Obese ob/ob mice with PAI-1-KO show decreased expression and secretion of TNFα in adipose tissue compared to obese mice expressing PAI-1 (Schafer et al. 2001). On the contrary, mice overexpressing PAI-1 have been suggested to exhibit reduced adipose tissue mass, lower fraction of stromal vascular cells in adipose tissue and lower fasting insulin concentration after high-fat diet-induced obesity compared to WT mice (Lijnen et al. 2003).

In humans, circulating PAI-1 concentration is increased in subjects with insulin resistance, abdominal obesity and T2DM compared to healthy subjects (Juhan-Vague et al. 1989, Pannacciulli et al. 2002). Circulating concentration of PAI-1 and adiponectin are inversely correlated in obese women, independent of BMI and visceral adiposity (Mertens et al. 2005). In severely obese subjects, plasma PAI-1 concentration and activity correlate with histologically determined liver fat content (Alessi et al. 2003). In addition, in subjects with non-alcoholic fatty liver, circulating PAI-1 concentration is higher compared to subjects without steatosis (Targher et al. 2005). Consistent with data of circulating PAI-1, its gene expression in adipose tissue is increased in obese compared to lean subjects (Alessi et al. 1997, Mavri et al. 2001).

TNFα increases the expression and secretion of PAI-1 in both subcutaneous and visceral human adipose tissue *in vitro* (Cigolini et al. 1999). Also IL-6 induces PAI-1 production by human adipocytes (Rega et al. 2005). Weight loss induced by bariatric surgery or by hypocaloric diet in obese subjects decreases significantly both plasma concentration and adipose tissue expression of PAI-1 (Primrose et al. 1992, Mavri et al. 2001). In humans, contrary to mice and cell cultures (Samad and Loskutoff 1996), insulin appears to have no

effect on PAI-1 secretion in subcutaneous or visceral adipose tissue *in vitro* (Halleux et al. 1999). Further, *in vivo* euglycemic hyperinsulinemia does not have an effect on plasma PAI-1 concentration in normal subjects (Vuorinen-Markkola et al. 1992) or on adipose tissue interstitial PAI-1 concentration in obese subjects (Siklova-Vitkova et al. 2009).

Resistin

Resistin was first found in 3T3-L1 and mature mouse adipocytes and suggested to associate with obesity and to mediate insulin resistance in mice (Steppan et al. 2001, Rajala et al. 2003). Obese resistin-KO mice show decreased insulin resistance in the liver (Banerjee et al. 2004), muscle and adipose tissue (Qi et al. 2006), and decreased hepatic steatosis and VLDL production (Singhal et al. 2008) compared to WT mice. Overexpression of resistin results in hepatic and systemic insulin resistance (Satoh et al. 2004).

In humans, the role of resistin in obesity and insulin resistance is less certain (Arner 2005). The predominant cell type of resistin production appears to be monocytes and macrophages with no expression in myocytes or adipocytes (Savage et al. 2001, Patel et al. 2003). In humans, resistin increase IL-6, IL-8 and MCP-1 gene expression in adipose tissue (Nagaev et al. 2006) and TNFα, IL-1 and IL-6 expression in monocytes *in vitro* (Bokarewa et al. 2005, Silswal et al. 2005). Some *in vivo* studies in humans suggest relationship between circulating resistin concentration and obesity (Degawa-Yamauchi et al. 2003), insulin resistance (Hivert et al. 2008) and T2DM (Youn et al. 2004). Many, if not the most, of the relevant studies show, however, no such relationships (Furuhashi et al. 2003, Lee et al. 2003b, Chen et al. 2005b, Utzschneider et al. 2005). Interestingly, concentration of resistin in synovial fluid of inflamed joints is increased in patients with rheumatoid arthritis compared to patients with non-inflammatory joint diseases (Bokarewa et al. 2005).

In human macrophages, TNFα, IL-6 and LPS significantly increase gene expression and secretion of resistin while resistin expression is inhibited by glitazones and salicylates through inhibition of NFκB (Kaser et al. 2003, Lehrke et al. 2004). Also LPS-induced endotoxemia in humans increases circulating concentration and monocyte expression of resistin *in vivo* (Lehrke et al. 2004). Glitazones also decrease circulating resistin concentration in T2DM patients, and this decrease is associated with decrease in hepatic steatosis and insulin resistance (Bajaj et al. 2004). Weight loss of ~5 % by orlistat or sibutramine decreases resistin, and increase adiponectin serum concentrations (Valsamakis et al. 2004). Thus, although the role of resistin in human obesity and insulin resistance remains controversial, a relationship between resistin expression, macrophage infiltration and inflammation exists. This justifies further *in vivo* studies in humans.

2.4. 11β-hydroxysteroid dehydrogenase

In most peripheral tissues, two isozymes of 11β-hydroxysteroid dehydrogenase, type 1 and 2 (11β-HSD-1/2), are responsible for interconversion of hormonally inactive cortisone and active cortisol (Seckl and Walker 2001). 11β-HSD-1 converts cortisone into cortisol and 11β-HSD-2 *vice versa*. Isozyme type 2 is mainly expressed in the kidneys while the liver and adipose tissue express 11β-HSD-1.

In rodent adipose tissue, both adipocytes and SVF express 11β-HSD-1 (Napolitano et al. 1998). Obese rats show selective increase in 11β-HSD-1 activity in adipose tissue, and decrease in the liver (Livingstone et al. 2000). Selective inhibition (Alberts et al. 2003) or 11β-HSD-1-KO in mice (Kotelevtsev et al. 1997) enhances hepatic insulin sensitivity and protects from obesity. 11β-HSD-1-KO mice also have decreased gene expression of resistin and TNFα in adipose tissue compared to WT mice (Morton et al. 2004). Overexpression of 11β-HSD-1 selectively in adipose tissue in mice, essentially results in hyperlipidemia, visceral obesity, hepatic lipid accumulation, insulin resistance and diabetes (Masuzaki et al. 2001).

In humans, 11β-HSD-1 is expressed in both adipocytes (Bujalska et al. 2002) and mature monocyte-derived macrophages (Thieringer et al. 2001). Expression and activity of 11β-HSD-1 in adipose tissue are associated with BMI, waist circumference and measures of insulin resistance in most (Paulmyer-Lacroix et al. 2002, Lindsay et al. 2003, Wake et al. 2003, Kannisto et al. 2004) but not all (Tomlinson et al. 2002) studies. Activity of 11β-HSD-1 correlates with adipocyte cell size and appears higher in visceral compared to subcutaneous adipose tissue in humans (Bujalska et al. 1997, Veilleux et al. 2009). In spite of alterations in adipose tissue 11β-HSD-1 activity in obesity, circulating free cortisol concentration is comparable between obese and lean subjects (Strain et al. 1980). In healthy men, treatment with the non-selective inhibitor of 11β-HSD-1, carbenoxolone, increases whole body insulin sensitivity measured by euglycemic hyperinsulinemic clamp technique (Walker et al. 1995).

In human adipose tissue SVF cells *in vitro*, TNFα increases the activity and expression of 11β-HSD-1 while simultaneous insulin treatment inhibits these effects (Handoko et al. 2000). In human monocyte-derived macrophages *in vitro*, expression of 11β-HSD-1 is increased by LPS treatment (Thieringer et al. 2001). Both cortisol and insulin stimulate adipocyte differentiation during adipogenesis (Hauner et al. 1987). TNFα and different interleukins, such as IL-1 and IL-6, increase 11β-HSD-1 activity and/or gene expression in human adipocytes *in vitro* (Tomlinson et al. 2004). In humans *in vivo*, a 3-hour euglycemic hyperinsulinemia shows no effect on adipose tissue 11β-HSD-1 gene expression in healthy or T2DM subjects (Koistinen et al. 2004). Weight loss in obese subjects induced by hypocaloric diet, significantly decreased 11β-HSD-1 gene expression in subcutaneous adipose tissue (Purnell et al. 2009). Expression of 11β-HSD-1 in human monocyte-derived macrophages in obese and non-obese subjects has not been studied and the relationship between adipose tissue expression of 11β-HSD-1 and liver fat content is unknown.

2.5. Peroxisome proliferator-activated receptor γ

Peroxisome proliferator-activated receptors α, δ (or β) and γ (PPARα/δ/γ) are nuclear receptors that regulate transcription of vast array of genes (Lee et al. 2003a). PPARα is mainly expressed in the liver regulating FFA uptake and oxidation, and functions as a receptor for PPARα-agonist drugs, such as fibrates. PPARδ increases glucose uptake and insulin action in human muscle and its protein expression in muscle is upregulated in response to exercise (Fritz et al. 2006). PPARγ is mainly expressed in adipose tissue and macrophages with low expression in the liver, muscle and β-cells (Vidal-Puig et al. 1997, Semple et al. 2006). PPARγ plays an important role in adipocyte differentiation (Tontonoz et al. 1994) and in regulation of genes involved in FFA uptake and TAG synthesis.

Homozygous PPARγ-KO in mice is lethal but adipose tissue-specific KO results in adipocyte necrosis, decreased adipose tissue mass, compensatory increased adipocyte cell size (He et al. 2003) and infiltration of inflammatory cells (macrophages, lymphocytes) in adipose tissue (Imai et al. 2004). Additionally, these mice show increased FFA and TAG concentrations, decreased adiponectin and leptin concentrations in plasma, increased susceptibility to diet-induced fatty liver and insulin resistance in adipocytes and in the liver. Macrophage-specific PPARγ-KO in normal and lean mice has been shown to result in glucose intolerance, insulin resistance in skeletal muscle and in the liver and to blunted insulin-sensitizing effects of glitazones (Hevener et al. 2007).

The discovery of the glitazone class of insulin-sensitizing drugs that are selective agonists of PPARγ, has made it possible to study effects of PPARγ (Lehmann et al. 1995). Pioglitazone and rosiglitazone are currently available for treatment of hyperglycemia in T2DM. Glitazones lower circulating glucose and insulin concentrations both in the fasting and postprandial states, decrease circulating FFA concentration and ameliorate insulin sensitivity in adipose tissue, skeletal muscle and in the liver (Yki-Järvinen 2004). Beneficial effects on gene expression and secretion of adipocytokines (TNFα, IL-6, adiponectin, PAI-1, MCP-1), redistribution of visceral fat to subcutaneous depot, increased FFA uptake and TAG storage by adipose tissue and decreased hepatic and muscle fat content contribute to insulin-sensitizing actions of glitazones (Sharma and Staels 2007).

Data of PPARγ gene expression in human adipose tissue are controversial while different studies suggest decreased, unchanged or increased expression in obese compared to lean subjects (Auboeuf et al. 1997, Vidal-Puig et al. 1997, Sewter et al. 2002, Giusti et al. 2003). In human adipose tissue *in vitro*, PPARγ agonist decreases MCP-1 secretion (Bruun et al. 2003). In addition, in subjects with IGT pioglitazone decreases the number of histologically determined macrophages and expression of CD68 and MCP-1 in adipose tissue with concomitant increase in insulin sensitivity (Di Gregorio et al. 2005). Also in T2DM patients, glitazone treatment increases adiponectin and decreases TNFα, MCP-1, FFA and glucose plasma concentrations. In addition, glitazones increase adipose tissue expression of PPARγ and both whole body and hepatic insulin sensitivity in subjects with T2DM (Miyazaki et al. 2004, Tiikkainen et al. 2004).

Concerning regulation, fasting and experimental insulin-deficiency decreases, while high-fat diet and insulin administration increases adipose tissue expression of PPARγ in mice (Vidal-Puig et al. 1996). TNFα and IL-6 decrease expression of PPARγ in 3T3-L1 adipocytes (Rotter et al. 2003). In humans, insulin stimulates PPARγ expression in adipose tissue and adipocytes *in vitro* (Vidal-Puig et al. 1997), and 3-hour *in vivo* euglycemic hyperinsulinemia increases PPARγ expression in subcutaneous adipose tissue with no differences between control, obese or T2DM patients (Rieusset et al. 1999).

2.6. PPARγ coactivator 1α

The PPARγ coactivator 1α (PGC-1α) is a transcriptional cofactor that modulates transcriptional activity of PPARγ (Puigserver et al. 1998). PGC-1α is expressed mainly in the liver, brown adipose tissue, skeletal and heart muscles, but also in white adipose tissue. PGC-1α universally induces mitochondrial oxidative metabolism with additional tissue-specific effects (Handschin and Spiegelman 2006).

In humans, PGC-1α gene expression increases during preadipocyte differentiation and adipose tissue expression of PGC-1α correlates with that of IRS-1 and GLUT-4, and also with *in vivo* insulin action measured by euglycemic hyperinsulinemic clamp technique (Hammarstedt et al. 2003). Adipose tissue expression of PGC-1α is decreased in morbidly obese and insulin-resistant compared to lean and insulin-sensitive subjects (Hammarstedt et al. 2003, Semple et al. 2004). In T2DM patients, skeletal muscle PGC-1α expression is also decreased compared to healthy subjects (Patti et al. 2003). In the liver, fasting stimulates PGC-1α function and induces gluconeogenetic activity. The effects of acute regulation of PGC-1α expression by insulin in human adipose tissue in insulin-resistant and sensitive subjects have not been previously studied.

2.7. Lipid mediators of insulin resistance

2.7.1. Free fatty acids

The serum concentration of FFA in the fasting and postprandial states is increased in obese insulin-resistant compared to lean insulin-sensitive subjects (see section 2.1. in *REVIEW OF THE LITERATURE*). In humans, increase of serum FFA concentration induces whole body and muscle insulin resistance (Nuutila et al. 1992) in a dose-dependent and reversible manner (Boden and Chen 1995, Belfort et al. 2005). Acute elevation in circulating FFA concentration under insulin-deficient, but not under well-insulinized conditions, stimulates hepatic glucose production *in vivo* in normal subjects (Ferrannini et al. 1983). Increased FFA concentration also blunts suppression of hepatic glucose production by insulin in humans (Homko et al. 2003). In normal subjects after 16 to 24 hours of fasting, neither decrease nor increase in serum FFA by an antilipolytic drug, nicotinic acid, has an effect on EGP (Chen et al. 1999b). On the other hand, reduction of circulating FFA under hyperinsulinemic conditions by acipimox, an analog of nicotinic acid, augments the suppressive effect of insulin on hepatic

glucose production (Saloranta et al. 1991). An acute decrease in serum FFA concentration, induced by overnight administration of acipimox, enhances whole body insulin sensitivity in both obese non-diabetic and diabetic individuals (Santomauro et al. 1999).

Randle with colleagues were the first to suggest a biochemical mechanism for the FFA-induced defect in insulin-stimulated glucose uptake in muscle. The mechanism was studied using isolated heart and diaphragm muscles of rats and was called the glucose-fatty acid or Randle's cycle (Randle et al. 1963). The studies showed that oxidation of glucose and FA in muscle was reciprocally regulated, and the hypothesis was that FA oxidation results in mitochondrial accumulation of acetyl-coenzyme A (-CoA) that directly inhibits pyruvate dehydrogenase (PDH) and increases citrate concentration. Citrate inhibits phosphofructokinase (PFK) leading to cytosolic accumulation of G6P that can be used for glycogen synthesis but is also an inhibitor of hexokinase that is responsible for glucose phosphorylation in the initial irreversible step of glycolysis. PFK and PDH, on the other hand, are the essential enzymes in the later steps of glycolysis. Thus, the oxidation of glucose would be suppressed during increased oxidation of FA. This hypothesis prevailed long until it was questioned by *in vivo* studies in humans showing that increase in circulating FFA concentration increases neither citrate, G6P nor glucose concentrations in muscle cells (Boden et al. 1991, Roden et al. 1996, Dresner et al. 1999).

In rats, high-fat diet for 3 days increases fasting serum FFA concentration resulting in fatty liver and hepatic insulin resistance that could be attributed to increased activity of protein kinase C (PKC) and JNK and decreased activity of IRS-1 and -2 (Samuel et al. 2004). Another study on rat liver showed that increase in circulating FFA concentration activates PKC and NFκB in the liver and increases hepatic expression of TNFα and IL-1β and serum concentration of MCP-1 (Boden et al. 2005). MCP-1 may provide a link between expanding adipose tissue, inflammation and insulin resistance (see section 2.3. in *REVIEW OF THE LITERATURE*). Activation of PKC and NFκB can induce insulin resistance and inflammation in both muscle and the liver (Petersen and Shulman 2006). Also in 3T3-L1 adipocytes, FFA-induced insulin resistance was mediated by activation of IKK-β and PKC and by inhibition of IRS-1 (Gao et al. 2004). In mice, long-chain FFA induce activation of JNK and increase expression of IL-6 and TNFα in macrophages, while specific JNK-KO in bone marrow-derived cells (e.g. monocytes, macrophages) protects from high fat diet-induced insulin resistance and inflammation (Solinas et al. 2007). In addition, obesity and increased circulating FFA concentration induce ER stress that is accompanied with inflammatory responses and insulin resistance (Gregor and Hotamisligil 2007). In humans, increase in serum FFA concentration suppresses IRS-1 and PI3K activity in muscle cells decreasing GLUT-4 translocation and glucose transport (Dresner et al. 1999).

Thus, FFA have multiple metabolic effects beyond inhibition of glucose oxidation via substrate competition. The effect of intravenous high dose FFA to induce insulin resistance takes approximately 3 hours to develop (Boden et al. 1991), and different metabolites of FA

have been suggested to account for the defect in insulin action. These include DAG and ceramides that are discussed in detail in the following sections.

2.7.2. Diacylglycerols

After uptake into the cell, FA can be metabolized, depending on FA composition and the energy need of the cell, through three different pathways. FA are first esterified to fatty acyl-CoA and can then be β-oxidized in mitochondria, stored as TAG or metabolized into sphingolipids, such as ceramides (Holland et al. 2007b). DAG is formed *de novo* by combining two FA with glycerol-3-phosphate or by breakdown of phospholipids or TAG. DAG and ceramides are both able to inhibit insulin signaling, although the mechanisms are incompletely understood. HSL is considered crucial and rate-limiting lipolytic enzyme in DAG breakdown, since HSL-KO mice show accumulation of DAG in adipose and muscle tissue and decrease in stimulated lipolysis and glycerol release (Haemmerle et al. 2002).

Studies in animal muscle *in vitro* and *in vivo* suggest that increased serum FFA concentration increases intramyocellular accumulation of fatty acyl-CoA and DAG leading to inhibitory serine phosphorylation of IRS-1 by PKC (Yu et al. 2002a). This results in decreased PI3K activity and GLUT-4-mediated glucose uptake. In human muscle *in vitro*, treatment with saturated FA blunts insulin-stimulated glucose uptake and increases PKC activity (Montell et al. 2001). In addition, saturated FA tend to incorporate into DAG fraction.

In humans *in vivo*, expression and activity of muscle DAG kinase, an enzyme responsible for depleting DAG amount in cells, is decreased in T2DM patients compared to healthy subjects, thus suggesting a relationship between DAG and insulin resistance (Chibalin et al. 2008). In normal subjects, simultaneous euglycemic hyperinsulinemia and lipid (Intralipid) plus heparin infusion increased DAG content in muscle compared to insulin infusion alone (Itani et al. 2002). In the latter study, the increase in muscle DAG was accompanied by decreased amount of IκB and increased activity of PKC in muscle and decrease in whole body glucose disposal. These data suggest that DAG regulates insulin signaling and inflammation, which both contribute to insulin resistance.

2.7.3. Ceramides

Ceramides are sphingolipids with two FA of variable length linked to a sphingosine base. Ceramides can be synthesized in muscle and in the liver either *de novo* from saturated FA or from breakdown of sphingomyelin by sphingomyelinases (SMPD) (Holland and Summers 2008). *De novo* synthesis is heavily dependent on the supply of long-chain saturated FA that participate to the initial and rate-limiting step in the synthesis pathway (Merrill 2002). Ceramides function also as precursors for more complex sphingolipids (Figure 2).

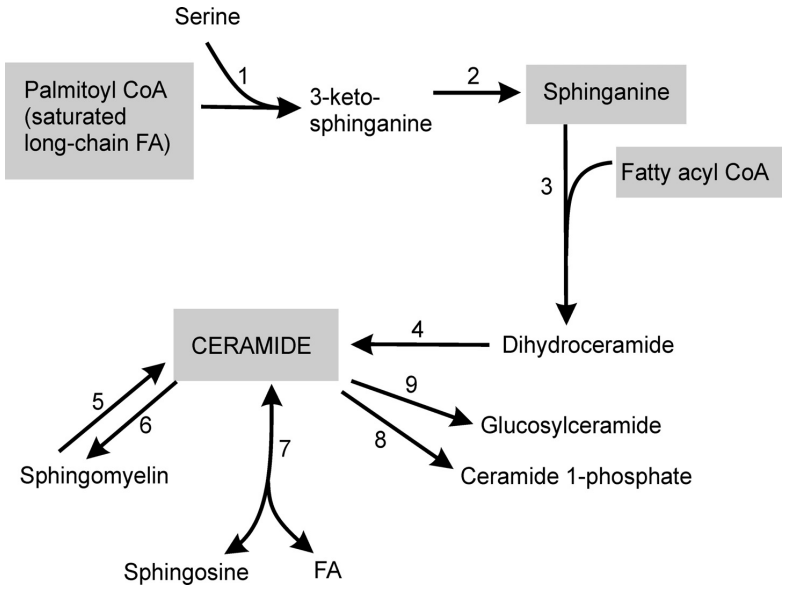

Figure 2. Flow chart of ceramide synthesis both *de novo* and from breakdown of sphingomyelin. Numbers indicate enzymes participating in sphingolipid metabolism; 1) Serine palmitoyltransferase, 2) 3-Ketosphinganine reductase, 3) Dihydroceramide synthase, 4) Dihydroceramide desaturase, 5) Sphingomyelinase, 6) Sphingomyelin synthase, 7) Ceramidase, 8) Ceramide kinase, 9) Glucosylceramide synthase. Modified from Holland and Summers 2008.

Although a minor component of all lipids (Kotronen et al. 2009c), ceramides function as bioactive lipid metabolites mediating insulin resistance and inflammation (Holland et al. 2007a) by inactivating Akt/PKB (Powell et al. 2003). Ceramides decrease GLUT-4 (Long and Pekala 1996) and increase TNF-α, MCP-1, PAI-1 and IL-6 (Samad et al. 2006) gene expression in 3T3-L1 adipocytes. Several studies show relationships between ceramides and different inflammatory pathways including TNFα-activated JNK/NFκB pathway and also toll-like receptor pathway that mediates the effects of LPS and stimulates inflammatory cytokine expression (Holland and Summers 2008) (Figure 3).

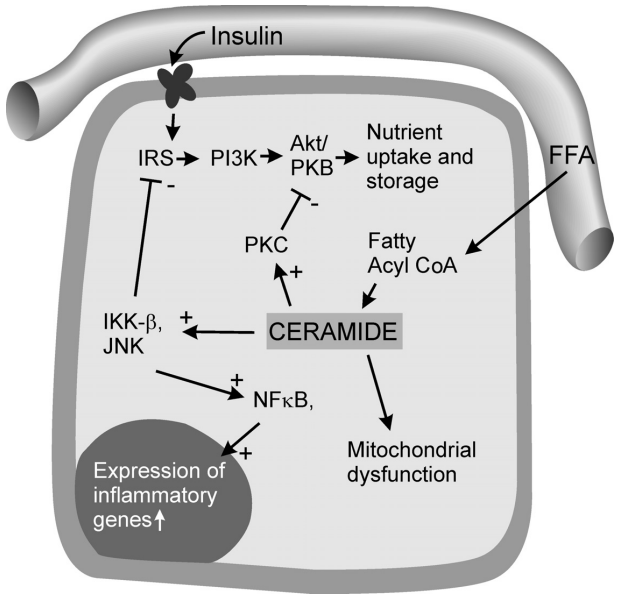

Figure 3. Regulation of cellular metabolism by ceramide. Arrows with (+) indicate stimulatory effect and lines with (-) indicate inhibitory effect. Modified from Summers 2006.

In muscle *in vitro,* insulin signaling is inhibited and ceramide formation increased by long-chain saturated FFA, but not by short-chain or unsaturated FFA (Chavez and Summers 2003). In rats, infusion of saturated lipids increases muscle ceramide content and whole body insulin resistance while infusion of unsaturated lipids results in insulin resistance independent of muscle ceramide accrual (Holland et al. 2007a). In cell cultures, TNFα stimulates both *de novo* and SMPD pathway of ceramide synthesis (Dbaibo et al. 2001). In animal models, inhibition of serine palmitoyltransferase (SPT), the essential enzyme regulating *de novo* ceramide synthesis, prevents the accumulation of ceramides in the liver and muscle and the decrease in insulin-stimulated glucose disposal in obesity or after lipid infusion (Holland et al. 2007a).

In humans, serum ceramides are carried in lipoproteins, mostly (~80 %) in VLDL and low-density lipoprotein (LDL) particles (Lightle et al. 2003, Kotronen et al. 2009c). After LPS-induced acute systemic inflammation in healthy subjects, concentration of ceramides in serum and in VLDL and LDL increased significantly. These results were reproduced in mice and, in addition, animal studies showed increased SPT activity in the liver and increased SMPD activity in serum suggesting that LPS-induced changes in lipoprotein ceramide content are due to increased *de novo* synthesis in the liver and increased breakdown of sphingomyelin in LDL particles (Lightle et al. 2003).

In human muscle, concentration of ceramides is increased in obese and insulin-resistant subjects compared to lean and insulin-sensitive subjects (Adams et al. 2004). Muscle ceramide content is also positively correlated with circulating FFA concentration. In healthy subjects, total ceramide content in muscle is, independent of BMI and serum FFA concentration, negatively correlated with insulin sensitivity measured with euglycemic hyperinsulinemic clamp technique (Straczkowski et al. 2004). In the latter study, lipid (Intralipid) plus heparin infusion increased ceramide content and SMPD activity in muscle and decreased whole body insulin sensitivity. Another study reported no differences in muscle ceramide content between T2DM or insulin-resistant subjects and insulin-sensitive subjects (Skovbro et al. 2008). Another study found no change in ceramide concentration in human skeletal muscle after 6 hours of lipid (Intralipid) infusion or acipimox treatment (Serlie et al. 2007).

In recent years, advances in lipidomics methodology (described in section 5. in *METHODS*) that combines ultra performance liquid chromatography, mass spectroscopy and data processing have enabled characterization and quantitation of numerous lipid species, such as TAG, DAG, FFA, ceramides and other phospholipids. FFA chain length and degree of saturation can also be determined. This method has not been previously used to study human adipose tissue.

3. Insulin resistance in the liver

3.1. Non-alcoholic fatty liver disease and insulin resistance

3.1.1. Definition

Sites of lipid deposition outside adipose tissue include the liver, skeletal muscle, heart muscle and the pancreas. NAFLD refers to increased (>5-10 % fat as determined by histology) liver fat content that is not due to excess alcohol consumption or any other known causes of liver disease (hepatitis B or C, toxic or autoimmune liver disease, hemochromatosis, Wilson's disease, hypobetalipoproteinemia) (Neuschwander-Tetri and Caldwell 2003). If assessed by non-invasive ^{1}H-MRS, liver fat of 5.6 % (55.6 mg of TAG/g of liver tissue) is the upper limit of normal. The latter threshold corresponds to the 95th percentile of liver fat distribution in healthy non-obese subjects (n=345) without a history of liver disease, excessive alcohol consumption or diabetes and with normal serum ALT activity in the Dallas Heart Study (Szczepaniak et al. 2005). NAFLD covers a spectrum of liver conditions from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis. The diagnosis of NASH requires a biopsy (Angulo 2009) and liver histology is characterized by lobular inflammation and hepatocellular ballooning in addition to steatosis.

3.1.2. Prevalence and significance

NAFLD is the most common cause of elevated serum aminotransferase activities (Clark et al. 2003). The prevalence estimates for NAFLD vary considerably depending on the methods and

population studied (Clark 2006). When liver fat was measured by ^{1}H-MRS in Caucasian, African American and Hispanic subjects (n=2349) in the population-based Dallas Heart Study, ~30-35 % had hepatic steatosis (Szczepaniak et al. 2005). The prevalence of NAFLD is even higher in obesity and T2DM (Angulo 2002). In morbidly obese patients (n=551) undergoing bariatric surgery, in whom liver biopsies where histologically analyzed, 86 % had steatosis, 24 % mild inflammation or NASH, and 2 % cirrhosis (Marceau et al. 1999). A normal serum ALT activity does not exclude steatosis since ~79 % of subjects with NAFLD have normal serum ALT (Browning et al. 2004).

NAFLD has been shown to predict CVD and T2DM in several prospective studies independent of obesity (Targher et al. 2007, Kotronen and Yki-Järvinen 2008). Simple steatosis can progress into more serious NASH with mild to moderate fibrosis in approximately 10-20 % of cases (Day 2006). Of these, ~5-15 % will develop severe fibrosis and of these, up to ~10 % cirrhosis over 15 years. The risk of hepatocellular carcinoma is also increased (Angulo 2002). It has been suggested that NASH might be the most common cause of liver transplantation by the year 2020 in the United States (Charlton 2004).

3.1.3. The metabolic syndrome and hepatic insulin resistance

The MetS, originally known as syndrome X (Reaven 1988), is a cluster of metabolically interrelated risk factors for CVD and T2DM. The prevalence of the MetS in adults in the United States and Europe is approximately 20-30 % (Grundy 2008). The MetS increases the risk of CVD events and death approximately 2-fold and the likelihood of developing T2DM 5-fold. Many previous definitions of the MetS exist and the most recent joint interim statement by several international organizations include five criteria: elevated waist circumference (≥94 cm in Europid males, ≥80 cm in females), elevated TAG (≥1.7 mmol/l) and glucose (≥5.6 mmol/l) concentrations, elevated blood pressure (systolic≥130 mmHg or diastolic≥85 mmHg) and reduced HDL-cholesterol concentration (<1.0 mmol/l in males, <1.3 mmol/l in females) (Alberti et al. 2009). In addition to defined thresholds, specific drug treatment for the four latter criteria is also sufficient to fulfill the criteria. To have the MetS, a subject has to have any three of the five risk factors (Alberti et al. 2009).

The prevalence of fatty liver is increased in obesity (Wasastjerna et al. 1972) and liver fat is significantly increased in subjects with the MetS (Alberti et al. 2005) than without. Liver fat content correlates closely with all components of the MetS and these correlations remain significant even after adjusting for BMI, age and gender (Kotronen et al. 2007b). In addition, other markers of insulin resistance, such as the intra-abdominal fat mass, and insulin and C-peptide concentrations, correlated positively with liver fat content (Westerbacka et al. 2004). Serum insulin and C-peptide concentrations are more closely related to liver fat content than to hepatic transaminase activities or components of the MetS (Kotronen et al. 2007b). These data imply that the non-alcoholic fatty liver is the hepatic manifestation of the MetS and it may help to distinguish the subjects who develop insulin resistance and the MetS, from those who do not.

Liver fat content correlates positively with hepatic insulin resistance that, in turn, plays a central role in the relationship between fatty liver and the MetS. When excess TAG deposits in the liver, the normal inhibitory action of insulin on hepatic glucose production is impaired (Ryysy et al. 2000, Seppälä-Lindroos et al. 2002) which, in turn, stimulates a compensatory secretion of insulin. This results in normal or mild hyperglycemia and hyperinsulinemia. Increased liver fat content also impairs hepatic insulin clearance that contributes to hyperinsulinemia (Kotronen et al. 2007a). Normally, insulin also inhibits secretion of TAG-rich VLDL by the liver (Malmström et al. 1997). Once the liver becomes fatty, this inhibition is blunted (Adiels et al. 2006) resulting in overproduction of VLDL and consequent hypertriglyceridemia, low serum HDL-cholesterol concentration and small dense LDL particles. This atherogenic lipid triad characterizes both the MetS and T2DM (Adiels et al. 2008).

3.1.4. Causes of liver fat and hepatic insulin resistance

Acquired causes. Obesity is associated with whole body and hepatic insulin resistance and liver fat, but the variation in liver fat content at any given BMI or waist circumference is considerable (Yki-Järvinen 2005, Kotronen et al. 2007b). Acquired obesity in monozygotic (MZ) twins is associated with increased liver fat content (Pietiläinen et al. 2005). Even a single mixed meal can rapidly increase liver fat content in normal subjects (Ravikumar et al. 2005). Fast food-based overfeeding in healthy subjects for 4 weeks resulted in ~9 % weight gain, 2- to 3-fold increase in liver fat content and 4- to 5-fold increase in serum ALT activity (Kechagias et al. 2008). Fatty liver is associated with increased intake of total and, especially, saturated fat (Tiikkainen et al. 2003, Pietiläinen et al. 2005). Weight loss decreases liver fat content (Luyckx et al. 2000, Petersen et al. 2005) and the decrease in hepatic TAG is relatively faster and greater than that from other sites in the body (Tiikkainen et al. 2003). Excess consumption of carbohydrates, especially fructose, has been associated with hypertriglyceridemia, insulin resistance and increased DNL in humans (Faeh et al. 2005, Havel 2005). In normal subjects, ingestion of fructose stimulates hepatic DNL more than glucose alone (Parks et al. 2008). Thus, increased consumption of refined sugars could contribute to the development of a fatty liver and the MetS. Concerning treatment of NAFLD, PPARγ agonists decrease liver fat content and increase hepatic insulin sensitivity in patients with T2DM, while metformin only ameliorates hepatic insulin sensitivity but does not appear to change liver fat content (Tiikkainen et al. 2004, Yki-Järvinen 2009).

Inherited causes. Family studies (Willner et al. 2001) and comparison between different ethnic groups (Browning et al. 2004, Petersen et al. 2006) suggest that genetic factors contribute to NAFLD. Studies comparing genotypes of different candidate genes, until the recent discovery of variation in adiponutrin gene (Romeo et al. 2008), have not provided conclusive evidence of an exclusive role of any gene. The heritability of liver enzymes has previously been assessed in adolescent or elderly twins but it is unclear whether these subjects had NALFD (Bathum et al. 2001, Middelberg et al. 2007).

Sources and composition of intrahepatocellular TAG. The sources of FA in intrahepatocellular TAG include adipocyte lipolysis, spillover of FFA from intravascular lipolysis of chylomicrons, hepatic uptake of chylomicron and VLDL remnants and DNL (see section 1.2.2. in *REVIEW OF THE LITERATURE)*. Although DNL is increased in human NAFLD (Donnelly et al. 2005), lipolysis of stored TAG in adipose tissue accounts for the major part of hepatic FA delivery. FA appear to originate mainly from peripheral tissues since even in markedly abdominally obese subjects, splanchnic lipolysis accounts only for up to ~30 % of hepatic FA delivery (Nielsen et al. 2004). The FA composition of intrahepatocellular TAG is characterized by increased amount of saturated and decreased amount of unsaturated FA (Kotronen et al. 2009b).

Role of adipose tissue in the pathogenesis of NAFLD. Adipose tissue is inflamed in obese subjects (Weisberg et al. 2003) and inflammation may induce insulin resistance in adipocytes that results in blunted antilipolytic effect of insulin (see sections 2.1 and 2.2. in *REVIEW OF THE LITERATURE)*. Adiponectin is secreted exclusively by adipocytes and suggested to possess anti-inflammatory and insulin-sensitizing effects and to decrease liver fat. Concentration of adiponectin in both serum and adipose tissue is decreased in obese and insulin-resistant subjects, and PPARγ agonist-induced decrease in liver fat content and increase in serum adiponectin concentration are closely interrelated in humans (see *Adiponectin* in section 2.3. in *REVIEW OF THE LITERATURE*). Thus, inflammation in adipose tissue and increased release of FFA and decreased production of adiponectin are likely to regulate liver fat and hepatic insulin sensitivity (see Figure 1 in section 2.2. in *REVIEW OF THE LITERATURE*). Human studies assessing the relationship between adipose tissue inflammation and NAFLD are scarse. In one study, the number of macrophages in omental, but not subcutaneous, adipose tissue was shown to be related to liver fat content in morbidly obese subjects (Cancello et al. 2006). Whether subcutaneous adipose tissue inflammation is associated with liver fat content in humans *in vivo* independent of obesity, is unknown.

AIMS OF THE STUDY

The present studies were performed to answer the following questions:

1) How does insulin *in vivo* acutely regulate expression of selected ”insulin resistance genes” (IL-6, TNFα, 11β-HSD-1, MCP-1, MIP-1α) and ”insulin sensitivity genes” (GLUT-4, PPARγ, PGC-1α, adiponectin) in subcutaneous adipose tissue of insulin-sensitive and insulin-resistant subjects (studies I and II)?

2) Is adipose tissue inflammation (i.e. infiltration of macrophages, expression of inflammatory genes) related to hepatic fat accumulation independent of obesity (studies III and IV)?

3) Are there differences in adipose tissue inflammation between equally obese groups of subjects with high compared to normal liver fat content (study IV)?

4) What are the relative roles of genetic and environmental factors on interindividual variation in serum ALT activity, a surrogate marker of liver fat content, in young adult MZ and dizygotic (DZ) twins (study V)?

SUBJECTS AND STUDY DESIGNS

1. Subjects

Baseline characteristics of the study subjects in studies I-V are shown in Table 2. The participants in studies I-IV were Caucasian women (total number of 55) who were recruited based on the following criteria: 1) age 18-60 years, 2) no known acute or chronic disease other than obesity determined by medical history, physical examination and standard laboratory tests (blood counts, sedimentation rate, electrolytes, glucose, creatinine, thyroid-stimulating hormone, electrocardiogram), 3) stable weight (±5kg) during the preceding 6 months, 4) alcohol consumption less than 20 grams (i.e. two drinks) per day assessed by interview and questionnaire, 5) no treatment with drugs that may alter glucose tolerance, 6) no pregnancy. Elevated serum liver enzyme activities (ALT; aspartate aminotransferase, AST; γ-glutamyl transferase, γGT) were not exclusion criteria.

For study V, subjects were recruited based on the same criteria as in studies I-IV from a population-based longitudinal study (FinnTwin16) of five consecutive birth cohorts (1975-1979) of Finnish twins, their siblings and parents through the National Population Registry of Finland (Kaprio et al. 2002). The subjects in study V were MZ and same-sex DZ twin pairs (total of 313 subjects) recruited based on their body weight, height and BMI at the last follow-up in the FinnTwin16 study (mean age 27.3±0.2 years, range 23.2-32.2). The aim was to cover the full BMI range of both normal-weight and obese subjects. The BMI in the present study subjects (mean 24.1±0.2 kg/m^2, range 17.6-42.9 kg/m^2) matched with that of the whole cohort (22.9±0.1 kg/m^2, 14.0-44.2 kg/m^2). Intrapair differences in liver fat content between 13 MZ twin pairs discordant for obesity (mean intrapair BMI difference 5.0±0.5 kg/m^2) was also separately determined.

The nature and potential risks of the studies were explained to all subjects before obtaining their written informed consent. The present studies adhere to the principles of the Declaration of Helsinki, and the Ethics Committee of Helsinki University Central Hospital approved the protocols.

Table 2. Baseline characteristics of the study subjects in studies I-V.

	Studies I and II		*Study III*		*Study III*		*Study IV*		*Study V*	
Variable	*Insulin-sensitive*	*Insulin-resistant*	*Non-obese*[A]	*Obese*[A]	*Non-obese*[B]	*Obese*[B]	*Normal liver fat*	*High liver fat*	*All*	*Subgroup*[C]
Number	11	10	10	10	7	7	10	10	313 (120 MZ, 193 DZ)	66 (46 MZ, 20 DZ)
Age (y)	32±3	40±3	34±3	39±4	41±3	41±3	44±3	37±2	27±0.1	26±0.2
Weight (kg)	69±4	90±4**	67±4	92±4***	64±3	97±6***	98±2	98±3	73±1	82±2
BMI (kg/m^2)	24.7±1.1	32.7±1.8***	24.2±1.0	33.1±1.7***	22.1±0.7	36.9±2.2***	35.4±1.1	36.7±0.8	24.7±0.3	27.7±0.7
W/H	0.86±0.001	0.91±0.01**	0.90±0.01	0.90±0.02	0.78±0.01	0.92±0.03**	0.98±0.06	0.97±0.03	0.86±0.004	0.90±0.01
Body fat (%)[D]	28±2	36±1***	27±2	37±1***	28±1	39±1***	40±1	37±1	29±1	33±1
Liver fat (%)	1.0±0.2	6.6±2.5*	0.8±0.1	6.8±2.5*	ND	ND	2.3±0.3	14.4±2.9***	ND	5.7±1.2
ALT (U/l)	23±3	33±7	23±4	34±6	16±2	27±4*	32±8	33±5	27±1	36±4
Glucose (mmol/l)	5.1±0.1	5.6±0.2**	5.2±0.1	5.5±0.2	4.6±0.2	5.1±0.3	5.1±0.2	5.5±0.2	5.0±0.03	5.4±0.06
HbA_{1c} (%)	5.0±0.1	5.5±0.1*	5.1±0.1	5.4±0.2	5.3±0.1	5.6±0.3	5.4±0.1	5.7±0.2	ND	ND
Insulin (mU/l)	3±0.4	10±1***	4±1	9±1**	3±1	11±2***	8±1	14±1**	6±0.2	8±1
LDL-chol (mmol/l)	2.2±0.1	3.1±0.1**	2.4±0.1	3.0±0.2	2.0±0.2	2.6±0.3	2.6±0.2	3.0±0.2	2.4±0.04	2.7±0.1
HDL-chol (mmol/l)	1.4±0.1	1.3±0.1**	1.8±0.1	1.4±0.1**	1.8±0.1	1.2±0.1**	1.5±0.1	1.2±0.1*	1.7±0.03	1.5±0.1
TAG (mmol/l)	0.8±0.1	1.4±0.2**	0.9±0.1	1.4±0.2*	0.7±0.1	1.6±0.2**	1.2±0.1	1.5±0.2	1.1±0.05	1.2±0.09

[A] These subjects underwent measurements of liver fat content, whole body insulin sensitivity and adipose tissue gene expression. [B] In these subjects, blood sample was taken to study gene expression both basally and after LPS-stimulation in monocyte-derived macrophages. [C] In this subgroup, liver fat content was measured using ^{1}H-MRS. [D] Percent body fat was measured by bioelectrical impedance method in studies I-IV and by dual-energy X-ray absorptiometry (DEXA) in study V. HbA_{1c}, glycosylated hemoglobin A_{1c}; chol, cholesterol; W/H, waist-to-hip ratio. Data are shown as mean±standard error of mean (SEM). *p<0.05, **p<0.01, ***p<0.001 for difference between the groups in studies I-IV.

2. Study designs

The specific design and measurements of the individual studies I-V are listed below.

Studies I and II. A total of 21 non-diabetic women were divided into insulin-sensitive and insulin-resistant groups based on their median whole body insulin sensitivity that was determined by euglycemic hyperinsulinemic clamp technique. Gene expression of GLUT-1, GLUT-4, PPARγ, PGC-1α, 11β-HSD-1, TNFα, adiponectin, IL-6, CD68, EMR1, ITGAM, ADAM8, MCP-1 and MIP-1α was measured in needle aspiration biopsy of abdominal subcutaneous adipose tissue using semi-quantitative real-time polymerase chain reaction (RT-PCR) technique. Biopsy was taken after an overnight fast and repeated after 3 and 6 hours of euglycemic hyperinsulinemia. In addition, adipose tissue protein concentration of MCP-1 and serum concentration of MCP-1 and MIP-1α were measured using enzyme-linked immunosorbent assay (ELISA) in study II. Body weight, height and BMI, as well as waist and hip circumference were measured. Percent body fat was determined with bioelectrical impedance analysis. Fasting blood sample for measurement of glucose, insulin, C-peptide and blood lipid concentrations was obtained.

Study III. A total of 20 non-diabetic women were divided into non-obese and obese groups based on their median BMI. Insulin sensitivity and adipose tissue gene expression after an overnight fast were measured as in studies I and II. Liver fat content was measured non-invasively using ^{1}H-MRS. Another group of 14 women was similarly divided into non-obese and obese groups and fasting blood sample was obtained for isolation and differentiation of circulating monocytes into macrophages *in vitro*. Gene expression of TNFα, 11β-HSD-1 and resistin in macrophages was measured both basally and after 3 and 6 hours of LPS-stimulation with RT-PCR. In all subjects, fasting blood sample was obtained and body fat and anthropometric measures were determined as in studies I and II.

Study IV. A total of 20 non-diabetic obese women were divided into two groups based on their median liver fat content measured by ^{1}H-MRS. Both groups were equally obese and of same age. Body fat composition (subcutaneous and visceral fat) was measured using magnetic resonance imaging (MRI). A surgical biopsy of abdominal subcutaneous adipose tissue was obtained after an overnight fast. Part of the biopsy was used for measurement of fat cell size by collagenase digestion and for quantification of inflammatory and macrophage marker gene expression with RT-PCR. Another part of the biopsy was used for immunohistochemical staining to assess the actual number of macrophages and necrotic adipocytes and their relations. Adipose tissue MCP-1 concentration was determined as in study II. To assess the numerous individual lipid species (such as ceramides, DAG and long-chain FA) in adipose tissue, a lipidomics analysis was performed using ultra-performance liquid chromatography and mass spectrometry. Fasting blood sample was obtained and anthropometric measures were determined as in studies I and II.

Study V. A total of 313 MZ (n=120) and same-sex DZ (n=193) twins participated in the study. Zygosity was confirmed by 10 informative genetic markers. Heritability of serum ALT activity and insulin concentration was estimated by heritability model fitting using Mx statistical package. To validate the use of ALT and insulin as surrogate markers of liver fat, ^{1}H-MRS was used to measure liver fat content in a subgroup of 66 subjects (46 MZ, 20 DZ subjects). The intrapair differences in liver fat content between MZ twin pairs (n=13) discordant for obesity (mean intrapair difference 5.0±0.5 kg/m^2) were also determined. Statistical analyses were corrected for clustered twin data. Fasting blood sample for measurement of liver enzyme activities and of insulin and glucose concentrations was obtained, and percent body fat was measured using DEXA. Anthropometric measures were determined as in studies I and II.

METHODS

1. Whole body insulin sensitivity

1.1. Euglycemic hyperinsulinemic clamp (studies I-III)

Whole body insulin sensitivity was measured using the euglycemic hyperinsulinemic clamp technique (DeFronzo et al. 1979). The study started at 07:30 after an overnight fast. Two 18 gauge catheters (Venflon, Viggo-Spectramed, Helsingborg, Sweden) were first inserted. One in an antecubital vein for infusion of insulin and glucose, and another retrogradely in a heated dorsal hand vein to obtain arterialized venous blood for measurement of plasma glucose concentration every 5 min and serum insulin concentration every 30 min. Regular human insulin (Insulin Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was infused in a primed-continuous fashion. The rate of the continuous insulin infusion was 1 mU/kg·min for 6 hours. Normoglycemia was maintained by adjusting the rate of a 20 % glucose infusion based on plasma glucose measurements. Whole body insulin sensitivity (M-value) was determined from the glucose infusion rate required to maintain normoglycemia (DeFronzo et al. 1979) between 30 and 360 min in studies I and II and between 30 and 120 min in study III. Glucose infusion rate is corrected for glucose pool size and the M-value is expressed as mg/kg fat free mass (FFM)·min.

2. Liver fat content

2.1. Proton magnetic resonance spectroscopy (studies I-V)

Localized single voxel (2x2x2 cm) proton spectra were acquired using a 1.5 T whole body system (Magnetom Vision, Siemens, Erlangen, Germany) that consisted of a combination of whole body and loop surface coils for radiofrequency transmitting and signal receiving. T1-weighted high-resolution MRI scans were used for localization of the voxel of interest. ^{1}H-MRS measurements of the liver fat were performed in the middle of the right lobe of the liver at a location that was individually determined for each subject avoiding vascular structures and subcutaneous fat tissue. Subjects lay on their stomachs on the surface coil that was embedded in a mattress to minimize abdominal movement due to breathing. The single voxel spectra were recorded using the stimulated-echo acquisition mode sequence with an echo time of 20 msec, a repetition time of 3000 msec, a mixing time of 30 msec and 1024 data points over 1000 kHz spectral width with 32 averages. Water-suppressed spectra with 128 averages were also recorded to detect weak lipid signals. A short echo time and long repetition time were chosen to ensure a fully relaxed water signal that was used as an internal standard. Chemical shifts were measured relative to water at 4.80 parts per million (ppm). The signal from methylene groups that represents intracellular TAG was measured at 1.4 ppm. Signal intensities were quantified by using an analysis program (VAPRO-MRUI, http://www.mrui.uab.es/mrui/). The spectroscopic intracellular TAG content (liver fat) was expressed as a ratio of the area under the methylene peak to that under the methylene and water peaks (×100=percent liver fat). This measurement has been validated against

histologically determined lipid content (Longo et al. 1993, Thomsen et al. 1994), and against estimates of fatty degeneration or infiltration by x-ray computer-assisted tomography by our group (Ryysy et al. 2000) and others (Thomsen et al. 1994). All spectra were analyzed by physicists who were unaware of the clinical data. The reproducibility of repeated measurements of liver fat in non-diabetic subjects studied on two occasions in our laboratory is 11 % (Sutinen et al. 2002).

3. Adipose tissue gene and protein expression and immunohistochemistry

3.1. Adipose tissue biopsy, fat cell size and total RNA and cDNA preparation (studies I-IV)

A needle aspiration (studies I-III) or surgical (study IV) biopsy of abdominal subcutaneous adipose tissue was taken under local anesthesia after an overnight fast (Yki-Järvinen et al. 1986). In studies I-III, the aspiration biopsy was repeated after 3 and 6 hours of hyperinsulinemia and each biopsy was obtained from a different location in the left, middle and right lower abdominal region. Part of the fresh basal sample was incubated with collagenase to isolate adipocytes and to measure cell size with light microscope (Hirsch and Gallian 1968). All other samples were immediately frozen in liquid nitrogen and stored at -80°C until ribonucleic acid (RNA) analysis. Frozen fat tissue samples (50-150 mg) were homogenized in 2 ml RNA STAT-60 (Tel-Test, Friendswood, TX) and total RNA isolated according to the manufacturer's instructions. After DNase treatment (RNase-free DNase set, Qiagen, Hilden, Germany), RNA was purified (RNeasy minikit, Qiagen) and the RNA concentration was measured using the RiboGreen fluorescent nucleic acid stain (RNA quantification kit, Molecular Probes, Eugene, OR) and Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). The quality of RNA was checked by agarose gel electrophoresis (Studies I-III) or by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) (Study IV). Isolated RNA was stored at -80˚C until qualification of the target messenger RNA (mRNA). A total of 0.1 µg RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, UK) and oligo $(dT)_{12\text{-}18}$ primers.

3.2. Quantification of gene expression (studies I-IV)

The concentration of mRNA of specific genes in adipose tissue was quantified by RT-PCR using the ABI 7000 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA). cDNA synthesized from 15 ng of total RNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and a gene-specific primer and probe mixture (pre-developed TaqMan Gene Expression Assays, Applied Biosystems) in a final volume of 15µl (studies I and II) or 25 µl (studies III and IV). All samples were run in duplicate. Relative expression levels were determined using a 5- to 6-point serially diluted standard curve generated from human adipose tissue cDNA and was expressed in arbitrary units. To compensate for differences in cDNA loading, gene expression was normalized

relative to the average expression of two separate housekeeping genes, ribosomal protein large P0 (RPLP0) and TATA-box binding protein (TBP). In study I, part of the gene expression quantification (β-actin (ACTB), PPARγ and adiponectin) was performed by RT-PCR using LightCycler technology (Roche Diagnostics, Mannheim, Germany) with LightCycler-FastStart DNA SYBR Green I Mix (Roche) and 0.5 μmol/l primers. An aliquot of 2 μl 1:10 diluted cDNA was brought to final volume of 20 μl and standard curves were created using purified cloned plasmid cDNA (QIAquick PCR Purification Kit, Qiagen) or specific PCR product. In study I, expression of PPARγ and adiponectin were expressed relative to housekeeping gene ACTB.

The assays used in studies I-IV were: Hs00154355_m1 for CD68, Hs00173562_m1 for EMR1, Hs00355885_m1 for ITGAM, Hs00174246_m1 for ADAM8, Hs00234140_m1 for MCP-1, Hs00234142_m1 for MIP-1α, Hs00605917_m1 for adiponectin, Hs00234592_m1 for PPARγ, Hs00194153_m1 for 11β-HSD-1, Hs00174131_m1 for IL-6, Hs00174128_m1 for TNFα, Hs00167155_m1 for PAI-1, Hs00609415_m1 for SMPD1, Hs00906924_g1 for SMPD2, Hs00218713_m1 for SMPD3, Hs00220767_m1 for resistin, Hs99999910_m1 for TBP and Hs99999902_m1 for RPLP0. Primer and probe sets for GLUT-1, GLUT-4 and ACTB have been published previously (Kannisto et al. 2004, Sutinen et al. 2004). The expression of a housekeeping gene is not affected by any studied variables or traits, and it is used as an endogenous reference to all other genes whose expression is measured. In studies I-IV, the expression of housekeeping genes ACTB, RPLP0 and TBP were essentially comparable between the studied groups.

3.3. Protein expression (studies II and IV)

A frozen sample of human subcutaneous adipose tissue (100-430 mg) was homogenized in lysis buffer (Ryden et al. 2002). The homogenate was centrifuged for 30 min (+4°C, 14000 rpm) and the supernatant was stored at -80°C until measurement of MCP-1 protein concentration using Human CCL2/MCP-1 ELISA Immunoassay kit (Quantikine, R&D Systems, Minneapolis, MN) and the Bio Assay Reader HTS 7000 Plus (Perkin Elmer, Norwalk, CT). Total protein was measured using the BC Assay-protein quantitation kit (Uptima Interchim, Montlucan, France).

3.4. Immunohistochemistry (study IV)

Part of the adipose tissue biopsies in study IV were used for immunohistochemical staining for the assesment of macrophages and necrotic adipocytes in adipose tissue. Positive CD68 staining was used as a marker for macrophages and negative perilipin staining as a marker for necrotic adipocytes, as previously described (Cinti et al. 2005). Staining was performed using a standard protocol on sections from formalin-fixed paraffin-embedded tissue blocks. Serial sections were microwave-treated in 10 mmol/l citrate buffer (pH 6.0) and incubated for 1 hour at room temperature with primary antibodies; mouse monoclonal anti-CD68 (Novocastra Laboratories, Newcastle upon Tyne, U.K.), guinea pig polyclonal anti-perilipin (Acris, Hiddenhausen, Germany) or mouse monoclonal isotypic control (Abcam, Cambridge, U.K.)

for CD68. After rinsing in PBS buffer containing 0.25 % Triton X-100 (pH 7.2), sections were incubated with secondary biotinylated goat anti-mouse (Dako cytomation, Dako, Glostrup, Denmark) or biotinylated goat anti-guinea pig (Abcam) antibodies. Avidin-biotin peroxidase complexes (Vector Laboratories, Burlingame, CA) were added followed by visualization with 3.3´-diaminobenzidine tetrachloride (Vector). All sections were counterstained with Harris hematoxylin (Histolab, Gothenburg, Sweden). For each subject, the number of macrophages (identified as CD68-positive cells) and CLS (perilipin-free adipocyte surrounded by at least three macrophages) (Cinti et al. 2005) within the entire section were counted by two independent observers using a light microscope and normalized for the total section area. Measurement of total section area and average adipocyte area (in arbitrary units) was performed using the GNU Image Manipulation Program 2.2 (GIMP 2.2).

4. Monocyte isolation, differentiation and stimulation (study III)

In study III, 50 ml of blood was drawn from 14 subjects after an overnight fast for the *in vitro* studies assessing basal and LPS-stimulated gene expression in monocyte-derived human macrophages. Monocytes were isolated from buffy coats by centrifugation in Ficoll-Paque (Amersham Bioscience AB, Uppsala, Sweden) gradient as described (Saren et al. 1996). Washed cells were suspended in Macrophage-SFM Medium (Gibco, Invitrogen Corporation, Grand Island, NY) containing streptomysin-penicillin (Sigma, St.Louis, MO). The monocytes were counted and seeded on 6-well plates. After 1 hour, non-adherent cells were removed and GM-CSF (5 ng/ml, Sigma) was added. Monocytes were cultured for 7 days for differentiation into macrophages. 10 µl of LPS (10 µg/ml, LPS of Salmonella typhimurium, Sigma) or saline (0.9 %) per 1 ml of SFM Medium/GM-CSF was added to stimulate cultured macrophages for 3 and 6 hours at +37°C in an atmosphere containing 5 % CO_2. Gene expression of TNFα, resistin and 11β-HSD-1 in macrophages was measured from cell lysates, as described for adipose tissue (see section 3.2. in *METHODS*). Saline, as a control, had no effect on macrophage gene expression at baseline or after 3 and 6 hours.

5. Lipidomics analysis (study IV)

Adipose tissue sample preparation. In study IV, lipidomics analysis of subcutaneous adipose tissue was performed. Approximately 20 mg of adipose tissue was weighed, and 20 µl of an internal standard mixture and 40 µl of saline (0.9 %) were added to the sample. Lipids were extracted from the samples with 200 µl of chloroform:methanol (2:1) solvent, and the tissue was homogenized with a glass rod. After vortexing for 2 min and incubating for 1 hour at room temperature, the lower layer (~100 µl) was separated by centrifugation at 10000 rpm for 3 min at room temperature. Labeled standard mixture (20 µl) was added to the lipid extract. Internal and external standards are listed online (http://dx.doi.org/10.2337/db07-0111).

Lipidomics analysis. Lipid extracts were analyzed on a Q-ToF Premier mass spectrometer (Waters, Milford, MA) combined with an Acquity Ultra Performance Liquid Chromatography (Waters). The column was an Acquity UPLC BEH C18 (10x50 mm) with 1.7 µm particles (Waters) and was kept at 50°C temperature. The binary solvent system A included water (1 %

1M NH_4Ac, 0.1 % HCOOH), and solvent system B included liquid chromatography/mass spectometer grade acetonitrile/isopropanal (5:2, 1 % 1M NH_4Ac, 0.1 % HCOOH) (Rathburn, Walkerburn, Scotland). The gradient started from 65 % A/35 % B, reached 100 % B in 6 min and remained there for the next 7 min. The total run time including a 5 min re-equilibration step was 18 min. The flow rate was 0.2 ml/min and injection volume 1 µl. The temperature of the sample organizer was set at 10°C. Lipid profiling was carried out using positive ion mode. The data were collected at mass range of charge to mass ratio 300-2000, with scan duration of 0.2 sec. The source temperature was set at 120°C, and nitrogen was used as desolvation gas (800 l/h) at 250°C. The voltages of the sampling cone and capillary were 39 V and 3.2 kV, respectively. Reserpine (50 µg/l) was used as the lock spray reference compound (5 µl/min), with a 10 sec scan frequency. The obtained data were converted into netCDF file format using Dbridge software from MassLynx (Waters). The converted data were processed using MZmine software version 0.60 (Katajamaa et al. 2006). Lipids were identified based on their retention time and charge to mass ratio using in-house built lipid database as previously described (Yetukuri et al. 2007). All the identified lipids were quantified by normalizing with corresponding internal standards. Sphingomyelins were normalized with GPCho(17:0/17:0) internal standard.

6. Body composition

6.1. Intra-abdominal and abdominal subcutaneous fat volumes (study IV)

Subcutaneous and intra-abdominal fat areas were determined using MRI. A series of 16 T1-weighted trans-axial scans were obtained from a region extending from 8 cm above to 8 cm below the 4^{th} and 5^{th} lumbar interspace (16 slice, field of view 375x500 mm^2, slice thickness 10 mm, breath hold repetition time 138.9 msec, echo time 4.1 msec). Fat areas were measured using an image analysis program (Alice 3.0, Parexel, Waltham, MA). A histogram of pixel intensity in the intra-abdominal region was displayed, and the intensity corresponding to the nadir between the lean and fat peaks was used as a cut-off point. Intra-abdominal adipose tissue was defined as the area of pixels in the intra-abdominal region above this cut-off point. Subcutaneous adipose tissue area was defined after manually drawing the demarcation of subcutaneous and intra-abdominal adipose tissue.

6.2. Whole body fat content (studies I-V)

In studies I-IV, percent whole body fat was measured using bioelectrical impedance plethysmography (BioElectrical Impedance Analyzer System model number BIA-101A, RJL Systems, Detroit, MI) (Lukaski et al. 1985). In study V, percent body fat was measured using DEXA (Lunar Prodigy, software version 2.15, Madison, WI) (Mazess et al. 1990).

6.3. Anthropometric indices (studies I-V)

Body weight and height were measured barefoot in light clothing to calculate BMI (weight (kg)/height $(m)^2$). Waist circumference was measured midway between the spina iliaca superior and the lower rib margin. Hip circumference was measured at the level of the greater trochanters.

7. Analytical procedures (studies I-V)

Plasma glucose concentration was measured in duplicate with Glucose analyzer II (Beckman Instruments, Fullerton, CA) using the glucose oxidase method (Kadish et al. 1968) (Studies I-V). Serum insulin concentration was measured using the Auto-DELFIA kit (Wallac, Turku, Finland) (Studies I-IV) or radioimmunoassay (Phadeseph Insulin RIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) (Study V). Serum C-peptide concentration was measured using radioimmunoassay method (Kuzuya et al. 1977). HbA_{1c} was measured using high-pressure liquid chromatography (Stenman et al. 1984) with fully automated Glycosylated Hemoglobin Analyzer System (BioRad, Richmond, CA) (Studies I-IV). Serum concentrations of total cholesterol, HDL and TAG were measured with enzymatic kits and an autoanalyzer (Roche Diagnostics Hitachi 917, Hitachi Ltd, Tokyo, Japan), and LDL using the formula of Friedewald (Friedewald et al. 1972) (Studies I-V). Serum FFA concentration was also measured using an enzymatic kit (Wako Diagnostics, Richmond, VA) and an autoanalyzer (Roche Diagnostics) (Study IV). Serum ALT, AST and γGT activities were measured as recommended by the European Committee for Clinical Laboratory Standards (Studies I-V). Specific ELISA kits were used to measure serum concentrations of adiponectin (B-Bridge International, San Jose, CA) (Study I), MCP-1 and MIP-1α (Quantikine, R&D Systems, Minneapolis, MN) (Study II).

8. Statistical analysis

8.1. Basic statistics (studies I-V)

In all studies, a p-value of less than 0.05 was considered statistically significant. The calculations were performed using GraphPad Prism (versions 3.0 and 4.0, GraphPad, San Diego, CA), SPSS 11.0 for Windows (SPSS, Chicago, IL) or Statview (SAS Institute, Cary, NC) (Studies I-IV). Statistical analyses on twin data (Study V) were performed using Stata statistical software (version 9.0, Stata Corporation, College Station, TX) and heritability analyses (Study V) using Mx statistical package (6^{th} edition 2003, Virginia Commonwealth University, Richmond, VA). Logarithmic transformation of variables with non-normal distribution was performed. In studies I-IV, physical and biochemical characteristics of the study subjects were analyzed using nonparametric methods. Data are shown as mean±SEM or mean with 95 % confidence intervals (95 % CI).

Studies I and II. Insulin-sensitive and insulin-resistant groups were compared using the nonparametric Mann-Whitney test. Effects of insulin were analyzed using Friedman's test followed by Dunn's post hoc test to compare single measurements. Correlations were calculated using Spearman rank correlation coefficient.

Study III. Obese and non-obese groups were compared using the Mann-Whitney test. Effects of LPS were analyzed using Friedman's test followed by Dunn's post hoc test to compare single measurements. Correlations were calculated using Spearman rank correlation coefficient. Multiple linear regression analysis was performed after logarithmic transformation of non-normally distributed data.

Study IV. The groups with normal or high liver fat content were compared using the Mann-Whitney test. Correlations were calculated using Spearman rank correlation coefficient.

Study V. For individual twins, the statistical analyses, significance tests and 95 % CI were corrected for clustered sampling of co-twins within pairs by using survey methods. Correlations were calculated using Pearson correlation coefficient. Analysis of covariance was used to compare slopes and intercepts of regression lines between liver fat and serum ALT and liver fat and insulin for men and women. If neither slopes nor intercepts differed between women and men, a common regression equation was calculated for all data. Wald test (a t-test adapted for clustered twin data) was used to compare independent variables between men and women, and MZ and DZ twins. Twin similarity within each zygosity group was assessed using intrapair correlations to provide initial evidence for familial aggregation and the presence of genetic effects.

8.2. Heritability analyses (study V)

The intrapair correlations and heritability estimates were calculated for all subjects together using gender-adjusted variables. Because of high correlation between BMI and serum ALT (r=0.44) and BMI and insulin (r=0.56), ALT and insulin values were also adjusted for BMI. Thus, the heritability estimates for serum ALT and insulin are independent of gender and BMI. The genetic variation can be divided into additive (A) genetic effects of individual alleles (correlation 1.0 for MZ and 0.5 for same-sex DZ co-twins) and to dominant (D) genetic effects by allelic interactions within a loci (correlation 1.0 for MZ and 0.25 for same-sex DZ co-twins). The environmental variation can be attributed to common (C) environmental effects shared by the co-twins (by definition, correlation 1.0 for both MZ and DZ co-twins) and to unique, individual-specific (non-shared), environmental (E) effects (uncorrelated between the co-twins). Models based on different combinations of these parameters can be generated; ADE, ACE, AE, DE, CE and E.

Our data include only twins reared together and do not therefore allow modeling of genetic dominance and common environmental effects simultaneously. In classical twin data, possible gene-environment interactions are estimated as part of the additive genetic component that may therefore also reflect genetic differences in susceptibility to environmental factors.

Further, one has to make the assumption of random mating which, if not present, would increase DZ correlations and thus inflate estimates of common environmental variance and reduce genetic variance (Silventoinen et al. 2003). The primary objective in heritability model fitting is to explain the observed data with a model that best balances parsimony and goodness of fit with as few parameters as possible (Neale and Cardon 1992). The model fitting starts with full ADE or ACE models, to which the fit of the submodels AE, DE, CE and E may be compared by using chi-square difference test statistics and degrees of freedom (d.f.) between nested models. The Akaike Information Criterion (AIC) is a global test of model fit combining the chi-square value and d.f.. Negative values of the AIC are considered as evidence for a better fit. From the best fitting model, it is possible to estimate the proportion of total variance attributable to A, D, C and E. Bivariate Cholesky decomposition parameterization (Neale and Cardon 1992) was calculated to determine whether the ALT, insulin and BMI shared genetic or environmental effects. This was estimated by correlations between genetic effects (r_A) and environmental effects (r_C, r_E) for the latter measures.

RESULTS

1. Acute effects of hyperinsulinemia on gene expression in adipose tissue (studies I and II)

Characteristics of the insulin-resistant and sensitive subjects in studies I and II are shown in Table 2 (see section 1. in *SUBJECTS AND STUDY DESIGNS*). Insulin-resistant subjects were more obese and had more body fat than lean insulin-sensitive subjects. Markers of insulin resistance, such as serum insulin, C-peptide and TAG concentrations were higher and HDL-cholesterol and adiponectin (12±1 mg/l in insulin-resistant vs. 18±2 in insulin-sensitive, $p<0.01$) concentrations lower in the insulin-resistant compared to sensitive group. Whole body insulin sensitivity was, by definition, over 2-fold higher in insulin-sensitive compared to resistant subjects (8.7 mg/kg FFM·min vs. 4.2, respectively, $p<0.001$). During the euglycemic hyperinsulinemia, serum insulin concentration was comparable between the groups (69±4 mU/l vs. 76±4, insulin-sensitive vs. insulin-resistant, non-significant, NS). Serum adiponectin concentration remained higher in the insulin-sensitive compared to resistant group during the insulin infusion (16±2 mg/l vs. 11±1, respectively at 3 hours; 16±2 vs. 11±1 at 6 hours, $p<0.01$ for both time points).

Gene expression in adipose tissue

GLUT-1, GLUT-4, PPARγ, PGC-1α and adiponectin. At baseline the adipose tissue expression of GLUT-1 (1033±543 vs. 722±251 arbitrary units, insulin-sensitive vs. insulin-resistant, NS), PPARγ and PGC-1α (Figure 4) were comparable between the groups. GLUT-4 and adiponectin expression were lower in the insulin-resistant compared to sensitive group (Figure 4). During hyperinsulinemia, GLUT-4 expression increased significantly in the insulin-sensitive group, whereas in the insulin-resistant group the increase was smaller and non-significant (Figure 4). GLUT-4 expression was significantly higher in the insulin-sensitive compared to resistant group at all time points. Expression of GLUT-4 and PGC-1 were also significantly correlated (data not shown). Adiponectin gene expression increased significantly during the insulin infusion only in the insulin-sensitive group and was significantly higher when compared to insulin-resistant group at all time points (Figure 4). The expression of PPARγ and PGC-1α increased significantly, but similarly, in both groups during the insulin infusion (Figure 4). The expression of GLUT-1 and the house-keeping genes (ACTB, TBP and RPLP0) were comparable between the groups at all time points (data not shown).

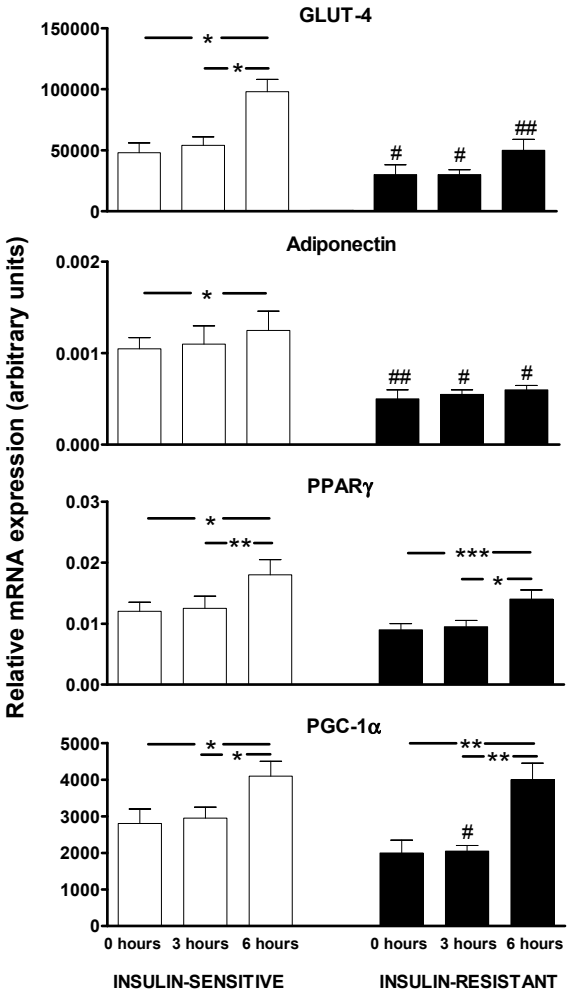

Figure 4. Gene expression of GLUT-4, adiponectin, PPARγ and PGC-1α in adipose tissue in insulin-sensitive (white bars) and insulin-resistant (black bars) subjects at baseline and after 3 and 6 hours of euglycemic hyperinsulinemia. *p<0.05, **p<0.01, ***p<0.001 for change between time points. #, p<0.05; ##, p<0.01 for difference between the groups.

11β-HSD-1, TNFα, IL-6, MCP-1 and MIP-1α. Before insulin infusion, 11β-HSD-1 gene expression was 2.4-fold higher in the insulin-resistant compared to sensitive group. Insulin further increased 11β-HSD-1 expression in the insulin-resistant group with no changes in the insulin-sensitive group (Figure 5). TNFα expression tended to be higher in the insulin-resistant compared to sensitive group at all time points with a significant difference at 6 hours. Insulin infusion had no significant effects on TNFα expression in neither groups (Figure 5).

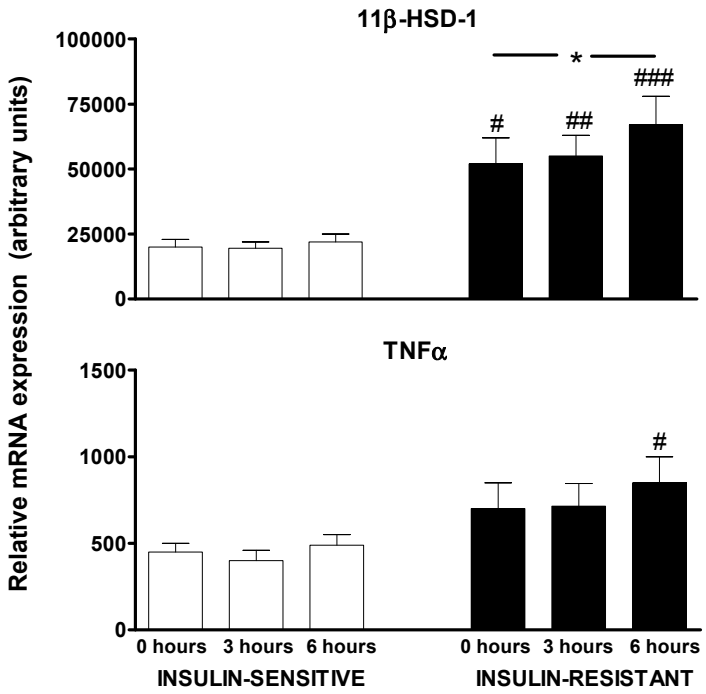

Figure 5. Gene expression of 11β-HSD-1 (upper panel) and TNFα (lower panel) in adipose tissue in insulin-sensitive (white bars) and insulin-resistant (black bars) subjects at baseline and after 3 and 6 hours of euglycemic hyperinsulinemia. *p<0.05 for change between time points. #, p<0.05; ##, p<0.01; ###, p<0.001 for difference between the groups.

IL-6 expression was comparable between the groups at baseline and increased significantly during insulin infusion in both insulin-sensitive and resistant groups. The expression of IL-6 was significantly higher in the insulin-resistant compared to sensitive group at 6 hours (Figure 6). Basally, gene expression of MCP-1 and MIP-1α were comparable between the groups. During insulin infusion MCP-1 expression increased more in the insulin-resistant (169±59 %) compared to insulin-sensitive (60±22 %) group (Figure 6). In the 8 control subjects who received saline infusion for 6 hours, expression of MCP-1 and MIP-1α remained unchanged (data not shown).

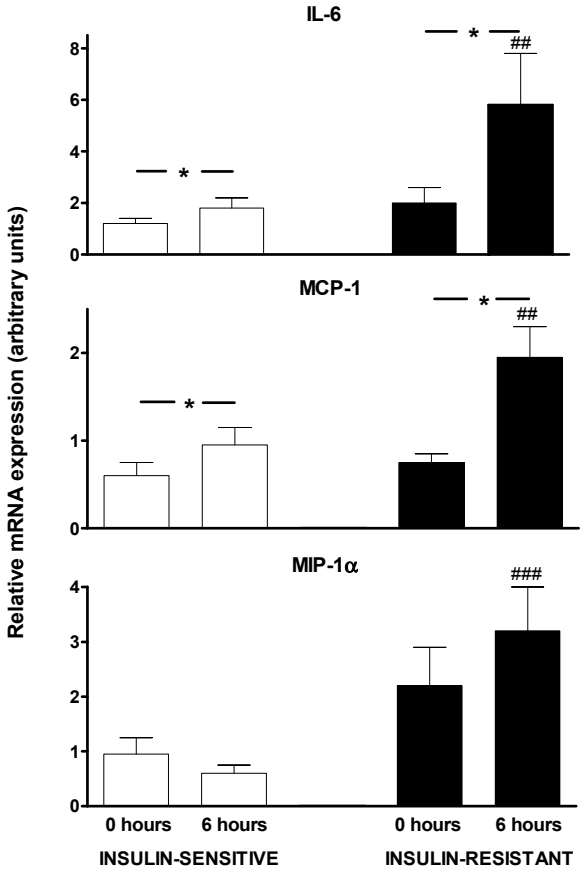

Figure 6. Gene expression of IL-6 (top panel), MCP-1 (middle panel) and MIP-1α (bottom panel) in adipose tissue in insulin-sensitive (white bars) and insulin-resistant (black bars) subjects at baseline and after 6 hours of euglycemic hyperinsulinemia. *p<0.05 for change between time points. ##, p<0.01; ###, p<0.001 for difference between the groups.

CD68, EMR1, ITGAM and ADAM8. At baseline, gene expression of ITGAM and ADAM8 were comparable between the groups, while the expression of CD68 and EMR1 were significantly higher in the insulin-resistant compared to insulin-sensitive group (Figure 7). At baseline, expression of both MCP-1 and MIP-1α correlated closely with that of CD68 (r=0.71, r=0.81, respectively, p<0.001 for both) and ITGAM (r=0.64, p<0.01; r=0.78, p<0.001), and the expression of MIP-1α also with that of EMR1 (r=0.55, p<0.01). Adipocyte cell size correlated significantly with the expression of CD68 (r=0.63, p<0.05).

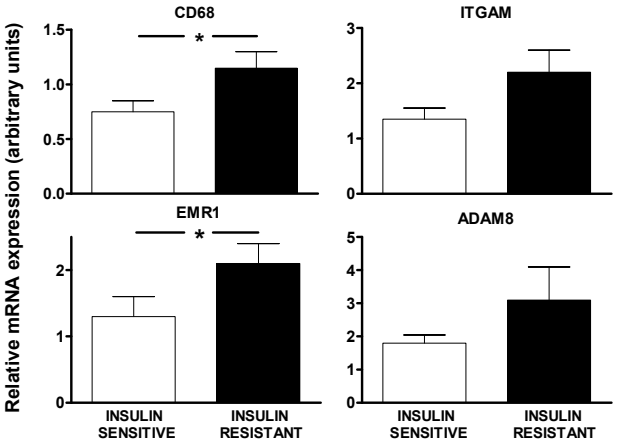

Figure 7. Gene expression of macrophage markers CD68 (upper left panel), ITGAM (upper right), EMR1 (lower left) and ADAM8 (lower right) in adipose tissue in insulin-sensitive (white bars) and insulin-resistant (black bars) subjects at baseline. *p<0.05 for difference between the groups.

Protein concentration of MCP-1 in adipose tissue and of MCP-1 and MIP-1α in serum

At baseline, adipose tissue and serum MCP-1 protein concentrations were comparable between the groups. Insulin increased significantly MCP-1 protein concentration in adipose tissue in insulin-resistant group with no change in the insulin-sensitive group (Figure 8). Insulin decreased serum MCP-1 concentration in the insulin-sensitive but not in the insulin-resistant group. At 6 hours, serum MCP-1 concentration was significantly lower in the insulin-sensitive compared to resistant group (Figure 8). As in study IV, protein concentration and gene expression of MCP-1 in adipose tissue correlated significantly (r=0.53, <0.001). Serum MIP-1α protein concentration was under the detection limit of the assay used (46.9 pg/ml) in all subjects and at all time points.

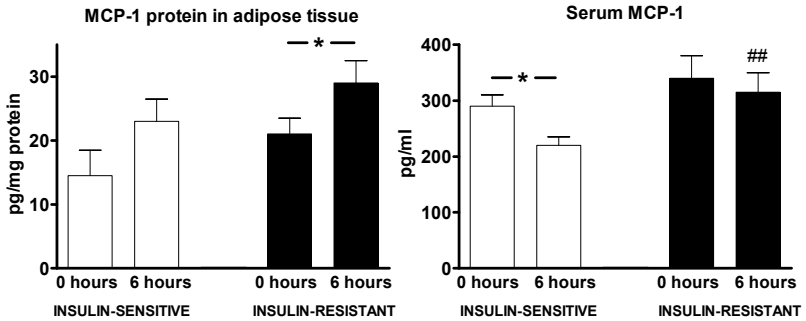

Figure 8. Concentration of MCP-1 protein in adipose tissue (left panel) and in serum (right panel) in insulin-sensitive (white bars) and insulin-resistant (black bars) subjects at baseline and after 6 hours of euglycemic hyperinsulinemia. *p<0.05 for change between time points. ##, p<0.01 for difference between the groups.

Relationships between adipose tissue gene expression and insulin sensitivity

Relationships between gene expression in adipose tissue and whole body insulin sensitivity (all subjects analyzed as one group) at baseline and after insulin infusion are shown in Table 3. Expression of adiponectin correlated with insulin sensitivity at baseline, and that of GLUT-4 at 3 and 6 hours. The expression of TNFα, 11β-HSD-1, IL-6 and MIP-1α correlated inversely with insulin sensitivity both basally and after 6 hours of insulin infusion. MCP-1 was negatively associated with insulin sensitivity only after insulin infusion. Adipose tissue expression of all monocyte/macrophage markers, except for ADAM8 at baseline, correlated inversely with insulin sensitivity both basally and after 6 hours of hyperinsulinemia (Table 3).

Table 3. Relationships between gene expression in adipose tissue and whole body insulin sensitivity (M-value) in all subjects (n=21) at baseline and after 3 and 6 hours of euglycemic hyperinsulinemia.

Gene	***0 hours***	***3 hours***	***6 hours***
Adiponectin	0.48*	-0.16	-0.24
GLUT-1	-0.08	-0.29	0.04
GLUT-4	0.44	0.66**	0.73***
PGC-1α	0.19	0.55*	0.34
PPARγ	0.14	-0.37	-0.14
IL-6	-0.48*	ND	-0.71***
MCP-1	-0.33	ND	-0.60**
MIP-1α	-0.62**	ND	-0.73***
TNFα	-0.44*	-0.53*	-0.46*
11β-HSD-1	-0.55*	-0.74***	-0.82***
ADAM8	-0.42	ND	-0.67**
CD68	-0.66**	ND	-0.68***
EMR1	-0.62**	ND	-0.56**
ITGAM	-0.50*	ND	-0.60**

*p<0.05, **p<0.01, ***p<0.001; ND, not determined.

2. Relationships between adipose tissue inflammation and liver fat content (study III)

Characteristics of the non-obese and obese subjects in study III are shown in Table 2 (see section 1. in *SUBJECTS AND STUDY DESIGNS*). Obese subjects had increased serum insulin and TAG, and decreased HDL-cholesterol concentration compared to non-obese subjects. Liver fat content was significantly lower (Table 2), whereas insulin sensitivity was higher (8.0±0.5 vs. 5.2±0.7 mg/kg FFM·min, respectively, $p<0.01$) in non-obese compared to obese subjects.

Gene expression in adipose tissue

Adipose tissue expression of CD68 (1.2±0.1 vs. 0.7±0.1 arbitrary units, obese vs. non-obese, $p<0.01$), ITGAM (2.3±0.4 vs. 1.3±0.2, $p<0.05$) and 11β-HSD-1 (0.6±0.1 vs. 0.3±0.1, $p<0.05$) were increased in obese compared to non-obese subjects. TNFα expression tended to be increased in the obese compared to the non-obese group, but did not reach statistical significance (1.4±0.3 vs. 1.0±0.2, obese vs. non-obese, NS). Expression of the housekeeping gene RPLP0 was comparable between the groups (data not shown).

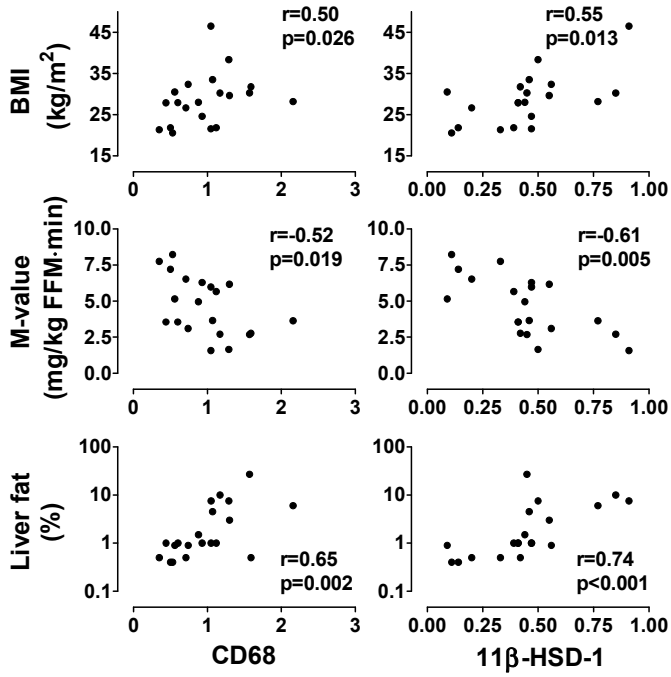

Figure 9. Relationships between adipose tissue expression (relative mRNA expression, arbitrary units) of CD68 and 11β-HSD-1 with BMI (top panels), with whole body insulin sensitivity (M-value) (middle panels) and with liver fat content (bottom panels). Spearman rank correlation coefficients (r) and p-values are shown.

Relationships between adipose tissue inflammation, liver fat content and insulin sensitivity

Expression of both CD68 and 11β-HSD-1 in adipose tissue were inversely correlated with whole body insulin sensitivity and positively correlated with liver fat content and BMI (Figure 9). Also adipose tissue expression of ITGAM correlated positively with liver fat content (r=0.67, p<0.01). Expression of CD68 correlated positively with that of TNFα and 11β-HSD-1 (Figure 10). The expression of two macrophage markers in adipose tissue, CD68 and ITGAM, were closely correlated (r=0.81, p<0.001). In multiple linear regression analysis, liver fat content significantly correlated with the adipose tissue expression of CD68, ITGAM and 11β-HSD-1, independent of BMI (Table 4).

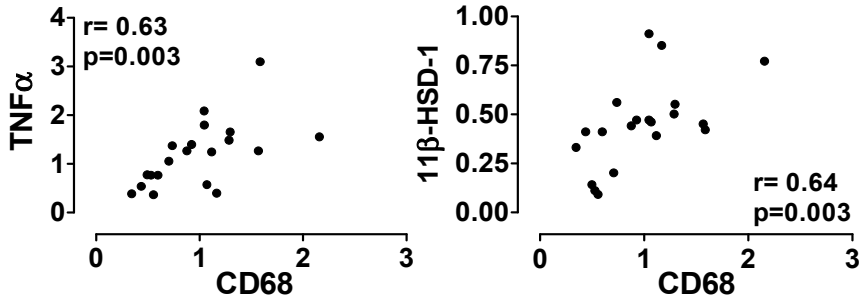

Figure 10. Relationships between adipose tissue expression (relative mRNA expression, arbitrary units) of CD68 and TNFα (left panel) and between CD68 and 11β-HSD-1 (right panel). Spearman rank correlation coefficients (r) and p-values are shown.

Table 4. Multiple linear regression analysis of correlates of liver fat content.

Dependent variable	*Independent variables*	*Regression coefficient±SEM*	*p-value (2-tail)*	*Squared multiple R ×100 (%)*
Liver fat (log)	BMI	0.038±0.01	0.016	58.3
	CD68	0.58±0.19	0.008	
Liver fat (log)	BMI	0.033±0.01	0.025	63.8
	ITGAM (log)	1.10±0.31	0.002	
Liver fat (log)	BMI	0.026±0.02	0.16	53.6
	11β-HSD-1	1.25±0.51	0.022	

log, logarithmically transformed

Gene expression in monocyte-derived macrophages

Expression of CD68 in human monocyte-derived macrophages was comparable between the obese and non-obese groups (1.2±0.1 vs. 1.2±0.1 arbitrary units, respectively, NS). After stimulation with LPS, expression of TNFα was significantly increased at 3- and 6-hour time points compared to basal in both groups, although expression decreased from 3- to 6-hour time point in the obese group (Figure 11). LPS had no effects on resistin expression in neither groups. 11β-HSD-1 expression increased after LPS stimulation significantly, but similarly, in both non-obese and obese groups (Figure 11). In control studies, no changes in macrophage gene expression were seen after administration of saline (data not shown).

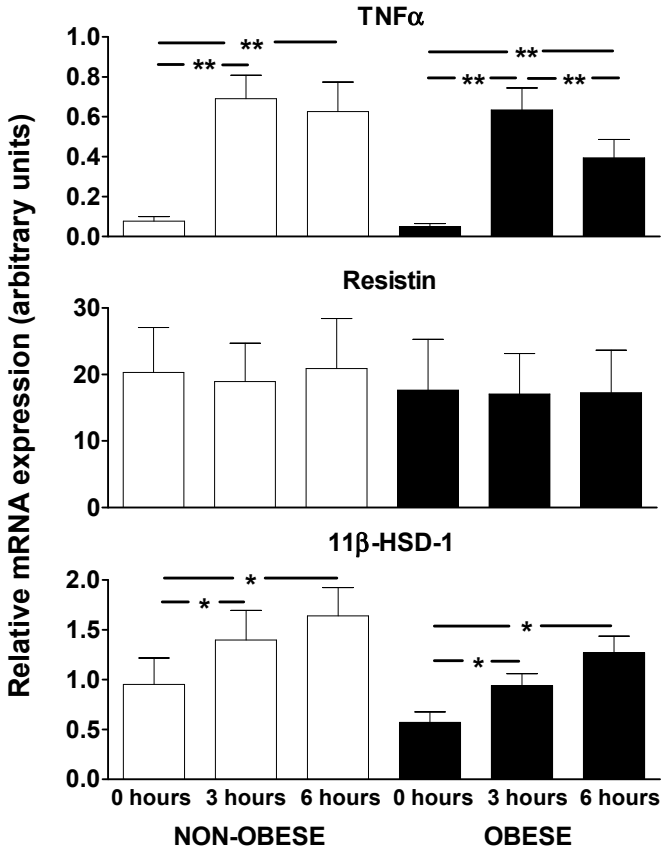

Figure 11. Gene expression of TNFα (top panel), resistin (middle panel) and 11β-HSD-1 (bottom panel) in human monocyte-derived macrophages at baseline and after 3 and 6 hours of LPS stimulation in non-obese (white bars) and obese subjects (black bars). *p<0.05, **p<0.01 for difference between time points.

3. Adipose tissue inflammation in equally obese groups of subjects with normal or high liver fat content (study IV)

Characteristics of the subjects with normal (mean 2.3±0.3 %, range 1-3.5 %) or high liver fat content (mean 14.4±2.9 %, range 6-34 %,) are shown in Table 2 (see section 1. in *SUBJECTS AND STUDY DESIGNS*). The groups were similar with respect to age, gender, BMI, and subcutaneous (7400±330 cm^3 in the normal and 6640±530 in the high liver fat group, NS) and intra-abdominal (1630±170 cm^3, 1980±260, respectively, NS) fat masses. The concentration of serum insulin was increased and that of HDL-cholesterol decreased in the high compared to the normal liver fat group (Table 2).

Gene expression in adipose tissue

Adipose tissue expression of CD68, MCP-1, MIP-1α and PAI-1 were significantly increased and that of adiponectin and PPARγ decreased in the high compared to the normal liver fat group (Table 5). Expression of 11β-HSD-1, IL-6 and TNFα did not differ significantly between the groups, although TNFα tended to be increased in the high liver fat group. Adipose tissue expression of sphingomyelinases SMPD1 (acid sphingomyelinase) and SMPD3 (neutral sphingomyelinase 2) were significantly increased in the high compared to normal liver fat group, while SMPD2 (neutral sphingomyelinase) showed only borderline significance (Table 5).

Table 5. The relative mRNA expression (arbitrary units) of different genes in adipose tissue in the normal and high liver fat groups.

Gene	***Normal liver fat***	***High liver fat***
PPARγ	1.1±0.1	0.9±0.1*
Adiponectin	1.0±0.1	0.9±0.1*
TNFα	1.5±0.3	2.4±0.8 (p=0.12)
11β-HSD-1	5.7±1.0	5.6±0.9
IL-6	0.1±0.03	0.1±0.02
PAI-1	2.8±0.5	4.0±0.6*
MCP-1	0.4±0.03	0.5±0.1*
MIP-1α	0.4±0.1	1.0±0.2**
CD68	1.9±0.1	3.2±0.4**
SMPD1	1.5±0.1	1.9±0.1**
SMPD2	1.1±0.1	1.3±0.1 (p=0.08)
SMPD3	1.3±0.1	1.8±0.2*

*p<0.05, **p<0.01 for differences between the groups. Data are shown as mean±SEM.

Expression of the macrophage marker CD68 correlated significantly with liver fat content but not with BMI (Figure 12, middle and right panels). As in study II, the protein concentration and gene expression of MCP-1 in adipose tissue correlated closely ($r=0.53$, $p<0.05$). Expression of the housekeeping genes RPLP0 and TBP did not differ between the groups (data not shown).

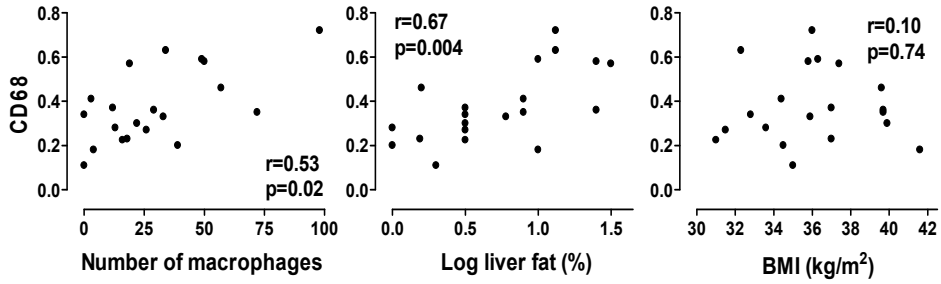

Figure 12. Relationships between adipose tissue expression (relative mRNA expression, arbitrary units) of CD68 and the number of macrophages (left panel), liver fat content (middle panel) and BMI (right panel). Spearman rank correlation coefficients (r) and p-values are shown.

Immunohistochemistry of adipose tissue

Positive staining for the macrophage marker CD68 was observed in 19 of the 20 adipose tissue samples. The total number of macrophages per section area did not differ between the groups, despite a tendency toward higher cell number in the high liver fat group (22±6 vs. 37±10, respectively, NS). The number of macrophages, however, was significantly correlated with the adipose tissue gene expression of CD68 in all subjects (Figure 12, left panel). Some macrophages were arranged in CLS around necrotic, perilipin-free, adipocytes (Figures 13 and 14). Adipose tissue expression of CD68 showed a borderline significant correlation with the number of CLS per section area in all subjects ($r=0.44$, $p=0.057$).

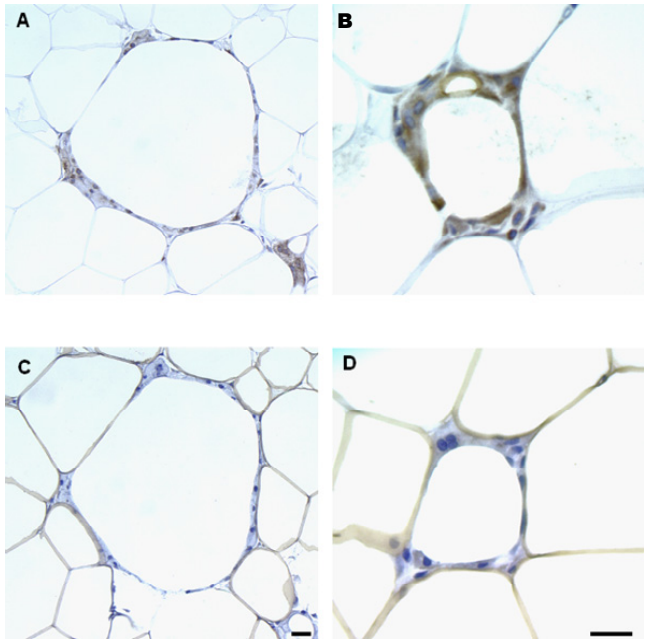

Figure 13. Light microscopy showing CLS in subcutaneous adipose tissue from an obese woman. Immunohistochemical staining for macrophage specific CD68 (panels A and B) is colored brown. Panels C and D show serial sections of A and B, respectively, that were stained for perilipin (brown). All sections were counterstained with hematoxylin (colored blue). Scale bar: 20 µm.

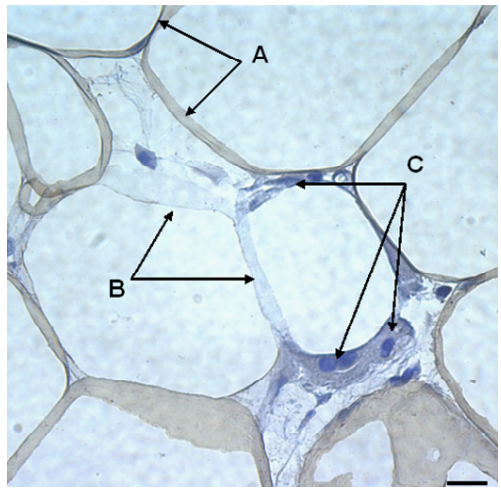

Figure 14. Light microscopy of subcutaneous adipose tissue from an obese woman stained for perilipin (colored brown). Dead (perilipin-negative) adipocytes are surrounded by macrophages forming a CLS. The blue-colored nuclei (C arrows) showed positive staining for the macrophage marker CD68 on a separate serial section (not shown). A arrows: Perilipin-positive staining (brown), B arrows: Perilipin-negative staining (light blue), C arrows: Macrophage (dark blue). Scale bar: 20 µm.

Adipocyte cell size

The average adipocyte cross-sectional area in the sectioned tissue and fat cell size measured by collagenase digestion did not differ between the normal and high liver fat groups (2067±109 vs. 2118±207 arbitrary units, respectively, NS for cross sectional area; 25.2±0.8 vs. 24.9±1.0 μm, respectively, NS for fat cell size).

Lipidomics of adipose tissue

A total of 154 lipid species were identified in adipose tissue and the differences in the high compared to normal liver fat group were dominated by increased ceramide, sphingomyelin and TAG concentration. No specific trend was observed for DAG. All 3 identified ceramide molecular species were increased in the high compared to normal liver fat group. The most abundant ceramide species, Cer(d18:1/24:1), showed significant 1.5-fold increase in the high liver fat group (Figure 15). The sphingomyelins were proportionally increased in the high liver fat group, although the most abundant sphingomyelin species SM(d18:1/16:0) did not differ between the groups (Figure 15**)**. Most of the 95 identified TAG species were increased in the high liver fat group and the fold change between the groups correlated positively with the total length of FA carbon chains in TAG (Figure 16). The degree of chain saturation of FA showed no such trend.

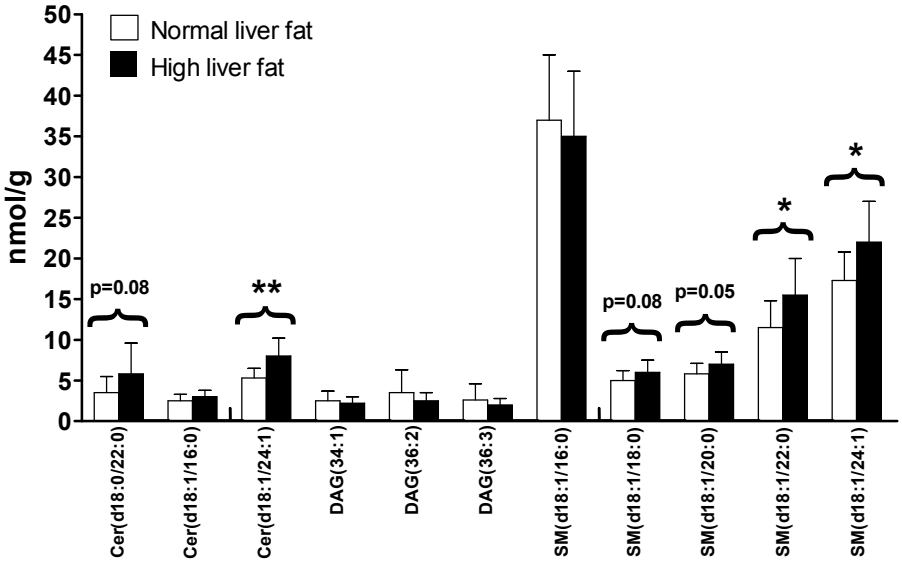

Figure 15. Lipidomics analysis of subcutaneous adipose tissue. Concentrations of detected ceramide (Cer), DAG and selected sphingomyelin (SM) species in the normal (white bars) and high liver fat (black bars) groups. *p<0.05, **p<0.01 for difference between the groups.

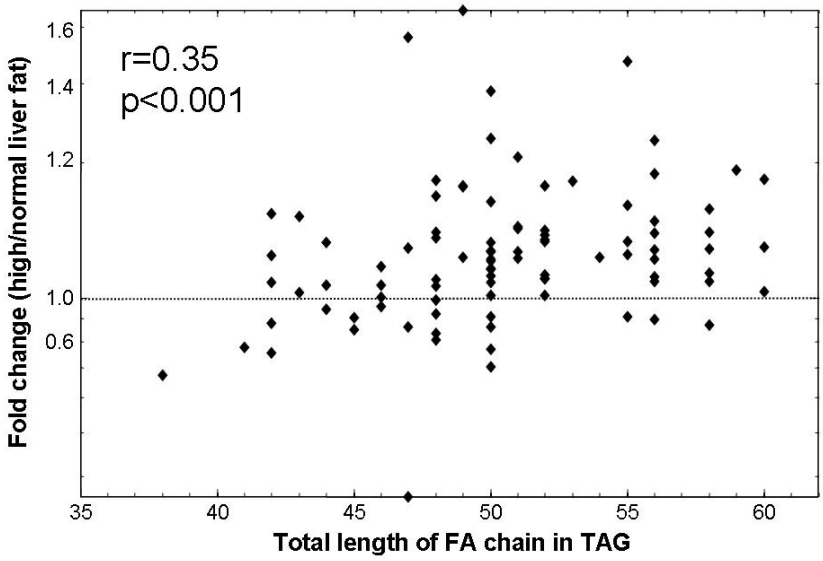

Figure 16. The correlation between total FA chain-length in TAG and the fold change between the high and normal liver fat groups. Spearman rank correlation coefficient (r) and p-value are shown.

4. Contribution of genetic factors to serum alanine aminotransferase activity, a surrogate marker of liver fat (study V)

Characteristics of the all 313 subjects and the subgroup of 66 subjects with liver fat measures are shown in Table 2 (see section 1. in *SUBJECTS AND STUDY DESIGNS*). Study group comprised 178 women and 135 men that were similar with respect to BMI (24.3±0.5 kg/m^2 vs. 25.3±0.4,respectively, NS), liver fat (4.7±1.7 % vs. 6.8±2.4, NS) and serum insulin concentration (6±0.3 mU/l vs. 7±0.5, NS). ALT (20±2 U/l vs. 37±2, p<0.001), AST (25±1 U/l vs. 29±1, p<0.001) and γGT (18±1 U/l vs. 28±2, p<0.001) activities were lower in women compared to men. The study group comprised 120 MZ twins (57 full pairs) and 193 DZ twins (88 full pairs). No differences in mean variances of the physical and biochemical characteristics between MZ and DZ twins were found (Table 6), and therefore the assumption of trait similarity between MZ and DZ twins for twin modeling was met. As expected, within-pair differences in BMI were greater in the DZ (4.6±0.4 kg/m^2, range 0.0-15.2) compared to MZ twins (2.7±0.4 kg/m^2, range 0.1-10.1, p<0.001).

Table 6. Characteristics of the MZ and DZ twins in study V.

Variable	*MZ*	*DZ*	*p-value*
Number	120 (57 full pairs)	193 (88 full pairs)	
Age (y)	27.1±0.3	27.4±0.2	0.38
BMI (kg/m^2)	25.3±0.6	24.4±0.3	0.20
Body fat (%)	30±1	29±1	0.43
ALT (U/l)	27±3	27±2	0.95
AST (U/l)	27±1	27±1	0.71
γGT (U/l)	21±2	23±2	0.48
Glucose (mmol/l)	5.0±0.1	4.9±0.04	0.18
Insulin (mU/l)[A]	6±0.4	6±0.4	0.98

[A] n=114 MZ, 187 DZ. Data are shown as mean±SEM

Relationships of serum ALT activity and insulin concentration with liver fat content

Serum ALT was strongly positively correlated with liver fat content in both women (r=0.70, 95 % CI 0.62-0.77, p<0.01) and men (r=0.50, 0.36-0.62, p<0.01) (Figure 17). A strong positive correlation was also found between liver fat content and serum insulin concentration with no difference between the genders (r=0.59, 0.40-0.73, p<0.001) (Figure 17). Both correlations remained significant also after adjusting for BMI (r=0.42, 0.32-0.51, p<0.001 for ALT; r=0.37, 0.27-0.46, p<0.01 for insulin). The correlation between liver fat content and BMI was r=0.47 (95 % CI 0.26-0.64, p=0.057).

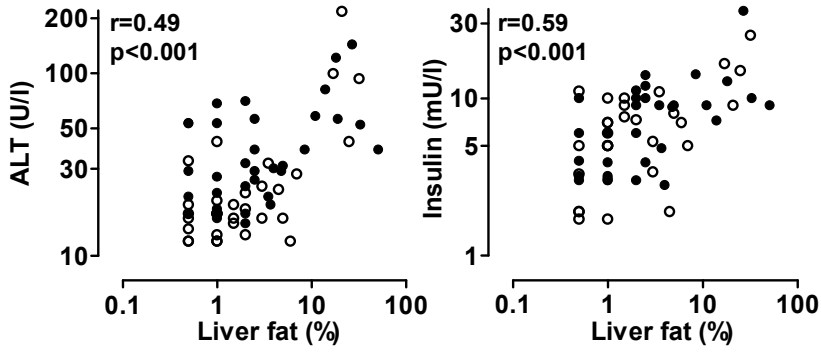

Figure 17. Relationships between liver fat content and serum ALT activity (left panel) and between liver fat content and serum insulin concentration (right panel) in all subjects (n=66). White circles denote women (n=32) and black circles denote men (n=34). Pearson correlation coefficients (r) and p-values are shown.

Intra-pair correlations for serum ALT activity and insulin concentration

Intra-pair correlations (twin A vs. twin B) were analyzed using BMI- and gender-adjusted variables. MZ twin pairs showed significantly higher intra-pair correlations for serum ALT compared to DZ twin pairs (r=0.65, 95 % CI 0.50-0.80, p<0.001 for MZ; r=0.04, 0.0-0.25, p=0.37, NS for DZ; p<0.001 for the difference between MZ and DZ) (Figure 18, upper panels) strongly demonstrating the influence of genetic factors. The intra-pair correlations for serum insulin tended to be higher for MZ compared to DZ twin pairs (r=0.58, 95 % CI 0.40-0.76, p<0.001 for MZ; r=0.34, 0.15-0.53, p<0.001 for DZ; p=0.076 for the difference between MZ and DZ) (Figure 18, lower panels). For serum ALT, the DZ correlations were less than half of the MZ correlations, suggesting that not only additive genetic but also possibly dominant genetic factors influence the trait. For serum insulin, the DZ correlations were more than half of the MZ correlations, suggesting the presence of shared environmental effects (e.g. within the family). Thus, the ADE model (see section 8.2. in *METHODS*) for serum ALT and the ACE model for serum insulin were used as starting points in heritability analyses.

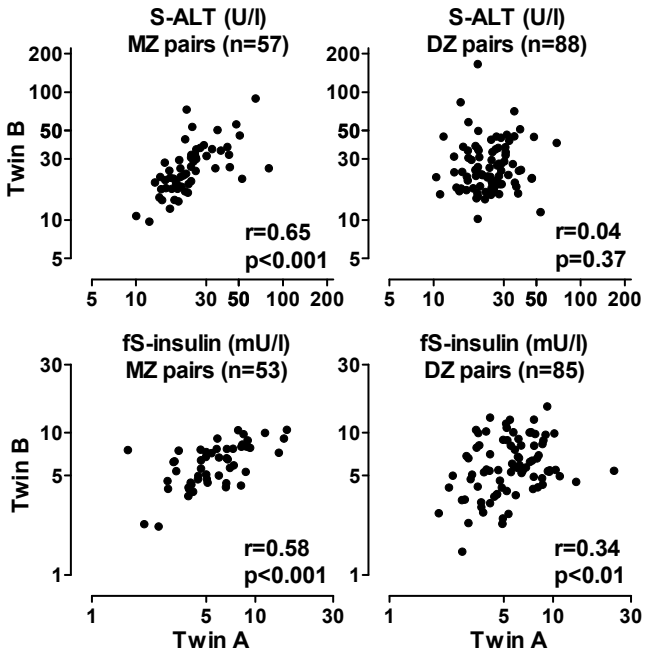

Figure 18. Intra-pair correlations between Twin A and Twin B in MZ and DZ twin pairs for serum ALT activity (upper panels) and serum insulin concentration (lower panels). Pearson correlation coefficients (r) and p-values are shown.

Heritability analyses for serum ALT activity and insulin concentration

The heritability estimates were analysed with combined data of both genders using gender- and BMI-adjusted values. For serum ALT, the ADE model fitted the data slightly better than the AE model (chi-square change p=0.035). For both models, AIC was low implying a good fit (AIC= -711.64 for the AE and -714.06 for the ADE model). In the ADE model, all genetic influence was placed on the D effect. Since dominant effects are rare in the absence of additive effects, the AE model was chosen as the final model for serum ALT. For serum insulin, the ACE model was used initially. The AIC was higher in the ACE (AIC=-664.87) model than in the AE model (AIC=-666.87). Dropping the C effect from the ACE model did not worsen the fit (p=0.97), and the point estimate for C effects in the ACE model was small and non-significant. This implies that shared family environmental effects for serum insulin are not significant and that the AE model fit the data the best.

AE models were used in the subsequent analyses. Cholesky bivariate decomposition analyses were performed to analyze the contribution of genetic and environmental effects, and their correlations on serum ALT and insulin and BMI. In a model where ALT and insulin were adjusted for BMI and gender, genetic effects (i.e. heritability) explained 55 % (95 % CI 36-70 %) of the variation serum ALT activity and 61 % (42-73 %) of that in serum insulin concentration. Contribution of environmental effects was 45 % (30-64 %) for serum ALT and 39 % (27-58 %) for serum insulin. Bivariate genetic modeling revealed that the correlation between additive genetic effects on serum ALT and insulin was r=0.30 (0.09-0.51), implying that 9 % (r^2=0.30×0.30) of the genetic factors influencing variation in serum ALT activity and insulin concentration are the same. No shared environmental effects explained the correlation between serum ALT and insulin when BMI was adjusted in the model.

Bivariate models for ALT and BMI, and for insulin and BMI were estimated. For serum ALT and BMI (adjusted for gender), the genetic correlation was r=0.39 (95 % CI 0.12-0.58) and the environmental correlation r=0.51 (0.30-0.68). For serum insulin and BMI (adjusted for gender), the genetic correlation was r=0.53 (31-0.68) and the environmental correlation r=0.64 (0.46-0.77). This implies that both serum ALT and insulin share genetic and environmental factors with BMI. The heritability for BMI was 68 % and, as mentioned above, 55 % for serum ALT activity and 61 % for serum insulin concentration.

Genetic and environmental effects on liver fat content

MZ pairs (n=23) with liver fat measures showed high intra-pair correlation for liver fat content (r=0.70, 95 % CI 0.49-0.92, p<0.001), but the number of DZ pairs with liver fat measures was too small to permit reliable heritability model fitting directly for liver fat content. Evidence of non-genetic effects was obtained from a correlation between intra-pair differences of liver fat content and those of BMI in MZ pairs (r=0.47, 0.07-0.74, p<0.01), implying that BMI differences explained 22 % of the differences in liver fat content. In a separate group of 13 obesity-discordant MZ pairs (BMI difference 5.0±0.5 kg/m^2), the obese

co-twins had significantly higher liver fat content compared to the non-obese co-twins (7.3±2.1 % vs. 2.0±0.4, respectively, p<0.01). The effects of acquired obesity on liver fat content, however, varied considerably between the individual twin pairs, and this could not be explained by BMI differences or by pair gender (Figure 19).

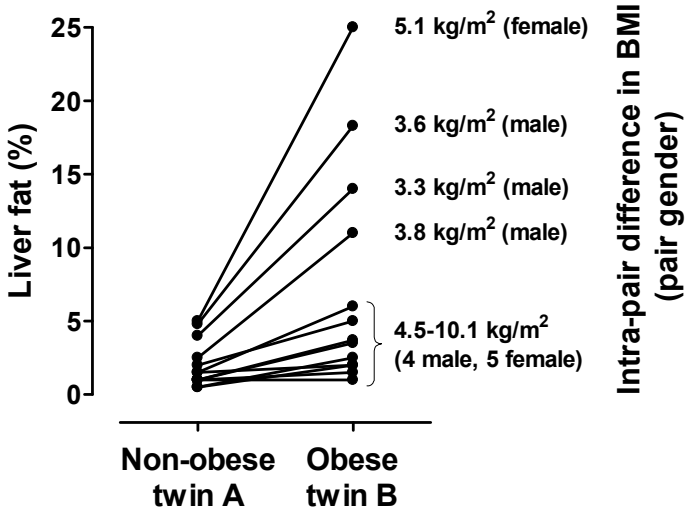

Figure 19. Effects of acquired obesity on liver fat content in 13 obesity-discordant MZ twin pairs. The intra-pair BMI difference and pair gender are shown.

DISCUSSION

1. Acute effects of hyperinsulinemia on gene expression in adipose tissue (studies I and II)

Studies I and II are among the first to compare the acute effects of *in vivo* hyperinsulinemia on genes considered to counteract (”insulin resistance genes”) or enhance (”insulin sensitivity genes”) insulin action in subcutaneous adipose tissue in non-diabetic insulin-sensitive and insulin-resistant subjects. These studies were the first to determine the acute insulin regulation of adipose tissue genes related to macrophage recruitment (MCP-1 and MIP-1α). The results showed that insulin-resistant subjects are not only characterized by failure of insulin to normally increase expression of ”insulin sensitivity genes” (such as GLUT-4 and adiponectin) but also by increased response of ”insulin resistance genes” (such as IL-6, 11β-HSD-1, MCP-1 and TNFα) to insulin when compared to insulin-sensitive subjects. Insulin also acutely increases expression of 11β-HSD-1, MCP-1 and PGC-1α in human adipose tissue, and whole body insulin sensitivity correlates inversely with adipose tissue gene expression of several monocyte/macrophage markers.

The study subjects were apparently healthy (except for obesity) women and were arbitrarily divided into more and less insulin-sensitive groups (10 insulin-sensitive and 11 insulin-resistant subjects, respectively) based on their median whole body insulin sensitivity. As subjects were randomly chosen from a group of wide range of obesity, it was expected that insulin-resistant subjects were more obese and had more body fat compared to insulin-sensitive subjects. Due to this approach in studies I and II, it is not possible to separate to which extent the different responses in gene expression to insulin are due to obesity *per se*. Thus, obesity may be a confounding factor when interpreting these relationships with insulin sensitivity. Adipocyte cell size is increased in obese insulin-resistant subjects and is suggested to impact adipose tissue gene expression (Coppack 2001). Abundant, but not totally consistent, data suggests differences in gene expression (Vidal 2001) and macrophage infiltraton (Cancello et al. 2006) between subcutaneous and visceral adipose tissue. In studies I and II, the repeated sampling of visceral fat in healthy subjects was not considered ethically or practically justifiable.

Classically, insulin resistance has been considered as a defective response of an insulin-sensitive gene, protein or pathway to insulin. The data from animal studies have suggested that this concept needs to be expanded because insulin not only regulates molecules and pathways that normally enhance insulin sensitivity but also molecules that normally induce insulin resistance or adipose tissue inflammation. TNFα (Iida et al. 2001), 11β-HSD-1 (Masuzaki et al. 2001, Thieringer et al. 2001), PAI-1, MCP-1 and sterol regulatory element binding protein 1c (SREBP-1c) (Sartipy and Loskutoff 2003) are examples of molecules that retain their sensitivity to insulin or even hyperrespond to insulin in insulin-resistant states. Studies I and II demonstrate this in human adipose tissue *in vivo*.

Several previous studies have compared the expression of the same genes as in study I between non-obese insulin-sensitive and obese insulin-resistant subjects in the basal state. These include reports of decreased expression of the ”insulin sensitivity genes” GLUT-4 in obese insulin-resistant (Garvey et al. 1991), PGC-1α in non-obese insulin-resistant (Hammarstedt et al. 2003) and adiponectin in obese and insulin-resistant subjects (Hu et al. 1996, Lihn et al. 2003). These findings were confirmed in study I. Deletion or overexpression of each of these genes has been shown to modulate insulin action in mouse models (Marshall and Mueckler 1994, Maeda et al. 2002, He et al. 2003, Koo et al. 2004). Reports of both decreased, unchanged and increased expression of PPARγ in obese compared to lean subjects have been published (Auboeuf et al. 1997, Vidal-Puig et al. 1997, Sewter et al. 2002, Giusti et al. 2003). In study I, basal expression of PPARγ was comparable between the insulin-resistant and sensitive groups as was the increase induced by *in vivo* insulin treatment.

PGC-1α is a transcriptional co-activator that increases gene expression of GLUT-4 and insulin sensitivity in skeletal muscle (Puigserver et al. 1998). It is absent from white adipose tissue in mice but expression in human adipose tissue (Hammarstedt et al. 2003) was confirmed in study I. Previous findings are extended by demonstrating in the present study that PGC-1α expression in adipose tissue *in vivo* is acutely increased by insulin infusion. Consistent with regulation of GLUT-4 by PGC-1α, adipose tissue expression of PGC-1α and GLUT-4 showed significant correlation.

Adiponectin expression increased significantly by the 6-hour time point in the insulin-sensitive but not in the insulin-resistant group. *In vitro* studies in 3T3-L1 adipocytes have reported both increase (Scherer et al. 1995) and decrease (Fasshauer et al. 2002) in adiponectin expression by insulin, and increase in adiponectin secretion in human omental but not subcutaneous adipocytes (Motoshima et al. 2002). Previous *in vivo* studies in humans found no change after 2.5 (Lihn et al. 2003) or 3 hours (Koistinen et al. 2004) of insulin infusion, consistent with the data at 3-hour time point in study I.

Concerning ”insulin resistance genes”, the reports of increased basal gene expression of 11β-HSD-1 in insulin-resistant compared to sensitive subjects (Paulmyer-Lacroix et al. 2002) are confirmed in study I. 11β-HSD-1 expression remained higher at all time points in the insulin-resistant compared to sensitive group, and increased in response to insulin in the former but not in the latter group. Previous data of insulin regulation of 11β-HSD-1 expression and activity are not unequivocal. In preadipocytes *in vitro*, insulin attenuates 11β-HSD-1 activity but synergizes with glucocorticoids to stimulate adipocyte differentiation with concomitant induction of 11β-HSD-1 activity (Stulnig and Waldhäusl 2004). In humans *in vivo*, consistent with the data at 3-hour time point in study I, hyperinsulinemia has been reported not to affect 11β-HSD-1 adipose tissue expression in healthy or T2DM subjects (Koistinen et al. 2004). 11β-HSD-1 is a gene abundantly expressed in macrophages in addition to adipocytes (Thieringer et al. 2001). Therefore, the increased expression of 11β-HSD-1 in the insulin-resistant group after 6 hours of insulin infusion in study I could also be due to insulin action targeted to macrophages.

In study I, basal expression of adipose tissue IL-6 was comparable between the groups. Like 11β-HSD-1, IL-6 was increased significantly more by insulin in the insulin-resistant compared to sensitive group. This change is most likely to occur in non-adipose cells, since isolated adipocytes account for only 10 % of total IL-6 release in human adipose tissue (Fried et al. 1998). IL-6 has been shown to downregulate GLUT-4, PPARγ and adiponectin expression in 3T3-L1 adipocytes (Bruun et al. 2003, Fasshauer et al. 2003b, Rotter et al. 2003), and to increase 11β-HSD-1 activity in primary cultures of human adipose stromal cells (Tomlinson et al. 2004). IL-6 is released systemically by adipose tissue (Mohamed-Ali et al. 1997) but the interstitial concentration of IL-6 in human adipose tissue is ~100 times higher than that in plasma (Sopasakis et al. 2004). These data suggest both paracrine and endocrine role for adipose tissue IL-6 in inducing insulin resistance (Rotter et al. 2003).

TNFα, in contrast to IL-6, is not secreted systemically by adipose tissue (Mohamed-Ali et al. 1997) and virtually all TNFα seems to originate from macrophages (Weisberg et al. 2003) or SVF cells (Fain et al. 2004a) in human adipose tissue. In a study examining the direct effect of insulin on macrophage gene expression using an array technique, insulin stimulated TNFα the most among all genes analyzed (Iida et al. 2001). *In vitro*, LPS has been found to increase TNFα production more robustly from human whole adipose tissue than from isolated adipocytes, while insulin had no effect on TNFα production (Sewter et al. 1999). In a human *in vivo* study using euglycemic hyperinsulinemic clamp, adipose tissue TNFα expression was transiently increased after 2 hours in a group of normal subjects (Krogh-Madsen et al. 2004). In study I, no differences in expression of TNFα between the groups were found in the basal state, whereas after 6 hours of insulin infusion the expression was increased significantly only in the insulin-resistant group. These data suggest that the response of TNFα expression to insulin is exaggerated in insulin-resistant subjects, although the increase by insulin failed to reach statistical significance. The mechanisms remain to be determined but may involve increased infiltration of macrophages in adipose tissue.

Gene and protein expression of MCP-1 at baseline are increased in TNFα-treated insulin-resistant 3T3-L1 adipocytes. Obese ob/ob and normal mice show increased adipose tissue expression of MCP-1 in response to *in vivo* insulin (Sartipy and Loskutoff 2003). In addition in mice, a transient increase in plasma concentration of MCP-1 after 1 hour of insulin injection was seen in obese mice while no changes was seen in WT insulin-sensitive mice (Sartipy and Loskutoff 2003). Consistent with these data, study II showed an exaggerated increase in MCP-1 gene and protein expression in adipose tissue in obese insulin-resistant as compared to lean insulin-sensitive subjects during insulin infusion. Gene and protein expression of MCP-1 in adipose tissue were also correlated significantly. Serum concentration of MCP-1 decreased significantly in the insulin-sensitive group, consistent with another saline-controlled study (Dandona et al. 2001), while there was no change in serum MCP-1 concentration in the insulin-resistant group. These data suggest that adipose tissue is not the main determinant of serum MCP-1 concentration in humans, and consistent with this, a catheterization study in humans found no systemic release of MCP-1 protein from subcutaneous adipose tissue (Dahlman et al. 2005).

Study II showed that adipose tissue gene expression of another macrophage chemoattractant adipocytokine, MIP-1α, is increased in obese insulin-resistant subjects compared to normal-weight subjects. Similar results were found in a subsequent study where adipose tissue MIP-1α expression, in addition to MCP-1, was increased in morbidly obese subjects compared to normal weight controls (Huber et al. 2008). Concerning circulating MIP-1α, a previous study using the same assay as in study II, found detectable serum concentration of MIP-1α in only 15 % of the T2DM patients studied (Meleth et al. 2005). MIP-1α serum concentration was under the detection limit (46.9 pg/ml) in all subjects in study II.

In the study reporting macrophage accumulation in adipose tissue in both rodent and human obesity, immunohistochemical detection and quantitation of CD68-expressing cells in subcutaneous adipose tissue showed that average adipocyte cell size and BMI were strong predictors of CD68-expressing cells (Weisberg et al. 2003). The increased basal expression of the macrophage marker CD68 in human adipose tissue in insulin-resistant subjects is confirmed in the present study, and an increase in another marker gene, EMR1, is reported. Increase in macrophage marker gene expression is likely to reflect an increase in macrophage number based on immunohistochemical data in previous studies (Weisberg et al. 2003) and in study IV. Significant correlation between adipose tissue expression of CD68 and adipocyte cell size was found in study II, consistent with previous data (Weisberg et al. 2003).

Studies I and II did not address the molecular mechanisms explaining how a gene can hyperrespond to insulin despite whole body insulin resistance that reflects insulin resistance mostly at the level of skeletal muscle (Nuutila et al. 1996). The multitude of genes involved in regulating insulin action and the discovery that adipose tissue of obese subjects contains inflammatory macrophages (Weisberg et al. 2003) also expressing insulin responsive genes, makes interpretation of the present data complex. Because of the repeated biopsies in studies I and II, it was considered unfeasible to take enough adipose tissue for separation of macrophages and adipose cells, and therefore it was not possible to locate the observed changes in gene expression to a given cell type. Theoretically, this implies that any of the following three scenarios might have taken place. First, genes hyperresponding to insulin could reside in a different cell type than those hyporesponding to insulin. For example, the hyperresponse of IL-6 to insulin that is expressed both in macrophages and adipocytes, could have occurred in macrophages, and the hyporesponse of adiponectin in adipocytes, the only cell type expressing adiponectin (Maeda et al. 1996). Second, both the hyper- and hyporesponse could have occurred in the same cell type but the increased basal or insulin-stimulated expression of ”insulin resistance genes” in the insulin-resistant group could have modulated the response to insulin. Third, a combination of both mechanisms might have taken place.

To conclude, acute insulin regulation of gene expression in adipose tissue of insulin-resistant subjects is characterized not only by hyporesponsiveness of ” insulin sensitivity genes”, such as GLUT-4, to insulin but also by hyperresponsiveness of ”insulin resistance genes” (IL-6, TNFα, 11β-HSD-1 and MCP-1). Although the attempts to ameliorate insulin resistance in

humans using anti-inflammatory approaches, such as TNFα antagonists, have been unsuccessful, the present studies show supporting evidence to continue efforts to develop such therapies.

2. Relationships between adipose tissue inflammation and liver fat content (study III)

The novel finding in study III was that adipose tissue inflammation, reflected as increased gene expression of macrophage marker genes and 11β-HSD-1, was associated with liver fat content in healthy non-diabetic non-obese and obese subjects. This relationship was also shown to be independent of obesity in multiple linear regression analysis. Human monocyte-derived macrophages showed no differences in expression of TNFα, 11β-HSD-1 or resistin between non-obese and obese subjects. Study III also confirms the increased expression of macrophage markers CD68 and ITGAM, and of 11β-HSD-1 in human adipose tissue in obese compared to non-obese subjects.

In keeping with most (Paulmyer-Lacroix et al. 2002, Lindsay et al. 2003, Wake et al. 2003, Kannisto et al. 2004), but not all (Tomlinson et al. 2002) previous studies, expression of 11β-HSD-1 was found to be increased significantly in subcutaneous adipose tissue of obese insulin-resistant subjects. Overexpression of 11β-HSD-1 selectively in adipose tissue in transgenic mice results in visceral obesity, insulin-resistance, diabetes and hyperlipidemia (Masuzaki et al. 2001). In adipose tissue, gene expression of 11β-HSD-1 correlates closely with both protein concentration (Kannisto et al. 2004) and enzyme activity of 11β-HSD-1 (Lindsay et al. 2003, Wake et al. 2003). As 11β-HSD-1 predominantly converts inactive cortisone (11-dehydrocorticosterone in mice) into biologically active cortisol (corticosterone in mice) *in vivo* (Seckl and Walker 2001), the increased expression of 11β-HSD-1 may increase local cortisol concentration in adipose tissue contributing to insulin resistance. The cell type responsible for 11β-HSD-1 overexpression in obesity cannot be determined from the data in study III.

11β-HSD-1-KO mice have lower expression of resistin and TNFα in adipose tissue than WT mice (Morton et al. 2004). Thus, one might have expected TNFα expression to be increased in adipose tissue in obese subjects. Also, the increased CD68 expression would be expected to be accompanied by increased TNFα expression. Although TNFα expression was increased, this increase did not reach statistical significance in study III. This could have been a type 2 error or, alternatively, due to phenotypic differences in macrophages. Activated M1-macrophages produce high amounts of proinflammatory cytokines such as TNFα and IL-6 while apoptotic cell engulfment appears to signal macrophages to transform into a reparative M2-population expressing anti-inflammatory properties (Gordon 2003, Bouloumie et al. 2005).

In study III, the expression of resistin in human macrophages (Patel et al. 2003) is confirmed, but no differences at baseline or after LPS-stimulation were found between obese and non-obese subjects. Resistin expression in adipose tissue or muscle does not differ between

insulin-resistant and insulin-sensitive subjects (Furuhashi et al. 2003, Lee et al. 2003b). Although resistin has proinflammatory properties and stimulates TNFα and IL-6 production in human peripheral blood mononuclear cells (Bokarewa et al. 2005), its role in human inflammation and insulin resistance is controversial. Increased infiltration of macrophages in adipose tissue in obesity may warrant, however, a role for resistin in inflammation and insulin resistance also in humans (Nagaev et al. 2006).

Expression of CD68 and the amount of CD68-positive cells are increased in obese adipose tissue (Weisberg et al. 2003, Xu et al. 2003b). In humans, expression of CD68 and ITGAM has not been previously related to liver fat content, or to expression of TNFα and 11β-HSD-1 in adipose tissue. These positive relationships may reflect an essential role of adipose tissue inflammation, rather than peripheral fat *per se*, in regulating hepatic insulin sensitivity, since liver fat (Sutinen et al. 2002) and the number of adipose tissue macrophages (Sievers et al. 2009) are increased even in patients with decreased subcutaneous fat mass.

Only two other human studies, one published before (Cancello et al. 2006) and the other after (Tordjman et al. 2009) study III, have examined the relationships between adipose tissue inflammation and liver fat. These studies found no relationship between the number of macrophages in subcutaneous adipose tissue and liver fat content in morbidly obese subjects undergoing bariatric surgery. However, the number of macrophages in visceral adipose tissue correlated with hepatic fibro-inflammatory lesions (Cancello et al. 2006) and with steatosis, fibrosis and inflammation (Tordjman et al. 2009). Healthy non-obese controls were not included in these studies. A limitation in study III is that gene expression of two macrophage markers was measured but immunohistochemical studies were not possible because a needle, rather than surgical, biopsy of adipose tissue was taken.

The studies showing macrophage infiltration in human and murine adipose tissue in obesity (Weisberg et al. 2003), and those showing that mice lacking IKK-β specifically in myeloid cells (i.e. monocytes, macrophages, neutrophils) are protected from insulin resistance in both skeletal muscle and the liver (Arkan et al. 2005), raised the interest in exploring the role of myeloid cells in the pathogenesis of insulin resistance in humans. Previous studies have suggested that insulin receptor tyrosine kinase activity is decreased in isolated monocytes from non-obese insulin-resistant compared to sensitive subjects (Frittitta et al. 1993), and that adhesion of human monocytes to endothelium *in vitro* is correlated with the degree of insulin resistance (Chen et al. 1999a). Freshly isolated circulating monocytes have been suggested to be in a proinflammatory state in obese subjects compared to non-obese controls (Ghanim et al. 2004). These data do not necessarily disagree with the findings in study III of unaltered basal and LPS-stimulated expression of TNFα, 11β-HSD-1 and resistin in human monocyte-derived macrophages, since *in vitro* differentiated macrophages, not freshly isolated monocytes, were studied. Obviously, this warrants measurement of a larger number of genes, as well as direct assessment of the macrophage fraction after isolation of different cell types in adipose tissue.

To conclude, study III demonstrates BMI-independent correlation between adipose tissue inflammation and liver fat content. Liver fat in turn is correlated with serum insulin and other signs of insulin resistance. These data may help to understand why some but not all obese subjects develop signs of insulin resistance.

3. Adipose tissue inflammation in equally obese groups of subjects with normal or high liver fat content (study IV)

Study IV was the first to determine whether increased liver fat content is associated with adipose tissue inflammation as determined by quantitative RT-PCR, immunohistochemistry and lipidomics in two equally obese groups of subjects with normal or high liver fat content. It was also the first study to apply lipidomics to analyze adipose tissue in humans. Several of the changes in expression of ”insulin resistance and sensitivity genes” that have been previously attributed to obesity, were also found to characterize subjects with high liver fat content independent of obesity. In addition, expression of CD68 correlated with the number of macrophages and CLS in adipose tissue. Study IV confirms that infiltrated macrophages surround perilipin-negative adipocytes to form CLS, as has been previously found in obese compared to lean subjects (Cinti et al. 2005). The lipidomics analysis of adipose tissue revealed increased concentration of ceramides, sphingomyelins and long-chain TAG in the high compared to normal liver fat group.

In study IV, adipose tissue expression of CD68, MCP-1, MIP-1α and PAI-1 were significantly increased and that of PPARγ significantly decreased in the high compared to normal liver fat group. Previous studies have documented increases in gene and protein expression of each of these genes in obese compared to non-obese subjects (Alessi et al. 1997, Di Gregorio et al. 2005, Lee et al. 2005, Mertens et al. 2005, Kim et al. 2006). The increased expression of MCP-1 is of particular interest since overexpression of MCP-1 in adipose tissue in mice is sufficient to induce macrophage infiltration in adipose tissue, systemic insulin resistance and fatty liver (Kanda et al. 2006). On the other hand, all these changes are ameliorated in high-fat fed mice with genetic deficiency of either MCP-1 (Kanda et al. 2006) or its receptor CCR2 (Weisberg et al. 2006). The cellular origin of MCP-1 was not investigated in study IV, as several studies have consistently shown macrophages to be the main source of this chemokine in human adipose tissue (Bruun et al. 2005, Fain and Madan 2005). MIP-1α is also involved in the recruitment and activation of macrophages and leukocytes (Maurer and von Stebut 2004). In study IV, expression of MIP-1α in human adipose tissue was increased in subjects with high compared to normal liver fat content.

The expression of PPARγ was significantly decreased in the high compared to normal liver fat group in study IV. Ablation of adipocyte PPARγ expression in mice has been shown to lead to adipocyte necrosis and infiltration of inflammatory cells in adipose tissue (Imai et al. 2004). Therefore, the reduced adipose tissue expression of PPARγ in the high liver fat group may have contributed to the increased inflammation in adipose tissue in study IV.

In addition to the reported increase in the infiltration of macrophages in adipose tissue of obese compared to non-obese subjects (Weisberg et al. 2003, Cancello et al. 2005), adipose tissue macrophages and perilipin-negative adipocytes arrange to form CLS in both obese mice and humans (Cinti et al. 2005). Perilipin is a protein located at the interface between the cytosol and TAG droplets in adipocytes and is involved in the regulation of lipolysis (Tansey et al. 2004). Using electron microscopy, negative perilipin staining was found in adipocytes with disrupted cell membrane, and that were partly engulfed by macrophages. It was therefore used as a marker of adipocyte death (Cinti et al. 2005). The immunohistochemical data in study IV confirmed the presence of CLS in human subcutaneous adipose tissue. The finding of increased macrophage infiltration and inflammation in adipose tissue independent of obesity in study IV is novel.

Fat cell size associates with obesity (Hirsch and Batchelor 1976, Coppack 2001), with the degree of macrophage infiltration in adipose tissue (Weisberg et al. 2003) and with adipocyte cell death (Cinti et al. 2005, Strissel et al. 2007). In HSL-KO mice, adipocyte cell size is markedly increased while body fat mass remains comparable with WT mice (Cinti et al. 2005). In these mice, adipose tissue is infiltrated with macrophages, suggesting that increased cell size rather than overall obesity is a trigger for macrophage infiltration. In study IV, the average adipocyte cell size and cross-sectional area were comparable between the groups. This suggests that cell size may not be the factor determining macrophage recruitment in adipose tissue of equally obese subjects with high as compared to low liver fat content.

Liver fat content can be increased by a high-fat diet containing plenty of saturated FA as compared to low-fat diet in humans (Westerbacka et al. 2005). Liver fat content also positively correlates with intake of both total and saturated fat in T2DM subjects (Tiikkainen et al. 2003), and in normal weight to obese healthy MZ twins (Pietiläinen et al. 2005). Long-chain saturated FA, with 16±1 carbons, are the main substrate for *de novo* synthesis of ceramides and other sphingolipids (Merrill 2002). Consistent with this, the lipidomics analysis in study IV showed significant increase in adipose tissue long-chain FA concentration in the high liver fat group. Thus, ceramides could contribute to the adverse metabolic effects of saturated fat.

Hydrolysis of sphingomyelin by SMPD could also have contributed to the increase in ceramide content (Summers 2006). Although enzymatic activity was not determined, gene expression of SMPD-1 and SMPD-3 were significantly increased in the high compared to normal liver fat group. The SMPD pathway is stimulated by several cytokines including TNFα (Dbaibo et al. 2001). In study IV, gene expression of TNFα in adipose tissue was 1.6-fold and marginally significantly increased in the high compared to normal liver fat group. Interestingly, SMPD activity is also found in atherosclerotic lesions (Marathe et al. 1999), and the ceramide content of LDL in arterial wall is higher than that of circulating LDL (Schissel et al. 1996). Thus, changes in inflamed adipose tissue containing macrophages bear some resemblance to those characteristic of atherosclerosis in the arterial wall. More recently, cross-sectional human studies have shown a correlation between serum ceramide and IL-6

concentrations in patients with coronary heart disease (De Mello et al. 2009). In addition, serum total ceramide concentration is increased in obese T2DM patients compared to lean and healthy subjects, and serum ceramide concentration correlates inversely with insulin sensitivity measured using euglycemic hyperinsulinemic clamp (Haus et al. 2009).

To conclude, study IV demonstrated that subjects with high liver fat content show increased inflammation and macrophage infiltration in subcutaneous adipose tissue compared to equally obese subjects with normal liver fat content. In addition, the concentration of ceramides and long-chain TAG are increased in subjects with high liver fat. Ceramides could contribute to saturated fat-induced insulin resistance in adipose tissue, although the cross-sectional design of study IV cannot prove causality.

4. Contribution of genetic factors to serum alanine aminotransferase activity, a surrogate marker of liver fat (study V)

In study V, heritability was high and significant for both serum ALT (55 %) and insulin (61 %). To validate the use of serum ALT as a surrogate marker of liver fat, it was measured by ^{1}H-MRS in a subgroup of 66 subjects. In keeping with previous findings in our group (Westerbacka et al. 2004, Kotronen and Yki-Järvinen 2008), significant and positive correlations between liver fat content and serum ALT and insulin were found. The high intrapair correlation for liver fat content in MZ pairs (r=0.70) was consistent with high heritability estimates for serum ALT and insulin.

The 313 subjects were recruited from a large population-based twin cohort comprising 4929 Finnish individual twins. The subjects in study V cover a wide range of BMI comparable with the whole cohort and represent healthy young adults who do not use excessive amounts of alcohol or regular medications. Medical history and daily alcohol consumption were carefully reviewed to exclude causes of steatosis or elevated liver enzymes other than those associated with obesity and insulin resistance. This enabled reliable estimation of genetic causes in variation of serum ALT activity and insulin concentration. Furthermore, in twin studies MZ and same sex DZ twins are perfectly matched for age, sex and ethnicity.

Heritability for ALT activity has previously been estimated in two twin studies. A Danish study investigated 290 pairs of elderly (age range 73 to 102 years) same-sex twins and found 35-61 % heritability for serum ALT, γGT, bilirubin, albumin and lactate dehydrogenase (Bathum et al. 2001). Heritability for serum ALT was 35 % and the best-fitting model was DE (35 % for dominant genetic and 65 % for unique environmental effects). The results did not change after adjusting for self-reported BMI and alcohol consumption. Information on medical history or medication status was not included in the analyses. In study V, almost twice as great heritability for serum ALT was found compared to the Danish study. In contrast to subjects in study V, the Danish twins were considerably older, their disease records or medications were not taken into account and BMI was self-reported. These factors can introduce variability that tends to decrease twin similarity and heritability estimates.

Another study included 965 Australian families with adolescent twins and their non-twin siblings to investigate the contribution of genetic and environmental factors to variation in serum ALT, AST and γGT activities, and in uric acid concentration (Middelberg et al. 2007). Genetic and environmental factors (e.g. diet, lifestyle) influencing these four parameters were studied longitudinally in the same twins at ages of 12, 14 and 16 years. The heritability of serum ALT was 40 %. The contribution of obesity, alcohol consumption, medications or disease records to heritability analyses were not reported in the latter study.

Previous studies have shown that serum insulin is a good surrogate marker for liver fat content, even independent of age, gender and BMI (Kotronen and Yki-Järvinen 2008). The fatty liver is insulin-resistant contributing to hyperinsulinemia (Ryysy et al. 2000, Seppälä-Lindroos et al. 2002) and impaired hepatic insulin clearance (Kotronen et al. 2007a). In line with these data, close correlation between liver fat content and insulin concentration was found in study V. The heritabilities for serum ALT activity (55 %) and insulin concentration (61 %) were of similar magnitude. These results suggest that genetic factors underlying interindividual differences in liver fat content may also regulate serum insulin concentration e.g. by changing insulin clearance and action or *vice versa*. Nonetheless, only a small fraction (9 %) of the genetic effects influencing serum ALT activity and serum insulin concentration were shared, indicating that these two surrogate markers of liver fat content are mainly determined by separate sets of genes.

Taken together, the twin data in study V suggest that genetic factors contribute significantly to variation in serum ALT activity, a surrogate marker of liver fat content.

Recently a single nucleotide polymorphism (SNP) (rs738409, C to G allele) in the human patatin-like phospholipase domain-containing 3 (PNPLA3) gene, encoding for adiponutrin, was found to be strongly correlated with increased liver fat content in African- and European-Americans and in Hispanics in the Dallas Heart Study (Romeo et al. 2008). This correlation has been confirmed in subsequent studies in Argentinian subjects (Sookoian et al. 2009) and in different European populations (Kantartzis et al. 2009, Kotronen et al. 2009a). The genetic variation in PNPLA3 did not, however, influence hepatic insulin sensitivity or serum FFA concentration (Kotronen et al. 2009a), or serum insulin concentration (Romeo et al. 2008, Kantartzis et al. 2009, Romeo et al. 2010). In addition, the same adiponutrin SNP has also been associated with increased transaminase activities (Yuan et al. 2008, Kollerits et al. 2010, Romeo et al. 2010). Adiponutrin is expressed in both adipose tissue and in the liver in humans, and is suggested to possess both lipolytic and lipogenic properties (Wilson et al. 2006), although its exact function is unknown to date. A recent study confirmed the TAG hydrolyzing ability of adiponutrin and showed that the rs738409 SNP results in intracellular TAG accumulation *in vitro* (He et al. 2010). These intriguing data warrant further studies to elucidate the role of adiponutrin in the pathogenesis of human NAFLD.

CONCLUSIONS

The present studies addressed the regulation of adipose tissue gene expression by insulin in insulin-sensitive and resistant subjects, and the interrelationships between adipose tissue inflammation and liver fat content in obese subjects with variable amount of liver fat. Results suggest that acute regulation of adipose tissue gene expression by insulin in insulin-resistant subjects is not only characterized by decreased response of genes related to insulin sensitivity but also by an increased response of genes related to insulin resistance and inflammation. Thus, impaired insulin action in adipose tissue may contribute to inflammation. Adipose tissue inflammation, defined by increased expression of inflammatory and macrophage marker genes, was found to correlate with liver fat content independent of obesity. The number of macrophages and expression of inflammatory genes, as well as concentration of ceramides and long-chain TAG, were increased in subjects with high liver fat content compared to equally obese subjects with normal liver fat. This suggests that increased liver fat content and adipose tissue inflammation are important determinants of insulin resistance even independent of obesity in humans. In addition, significant heritability for serum ALT activity was found suggesting that genetic factors contribute to variation in liver fat content in human NAFLD.

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ARTICLE

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Acute in vivo effects of insulin on gene expression in adipose tissue in insulin-resistant and insulin-sensitive subjects

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Abstract *Aims/hypothesis:* We determined the response of selected genes to in vivo insulin in adipose tissue in 21 non-diabetic women. *Materials and methods:* The women were divided into insulin-sensitive and -resistant groups based on their median whole-body insulin sensitivity (8.7±0.4 vs 4.2±0.3 mg kg^{-1} min^{-1} for insulin-sensitive vs -resistant group). Subcutaneous adipose tissue biopsies were obtained before and after 3 and 6 h of i.v. maintained euglycaemic hyperinsulinaemia. Adipose tissue mRNA concentrations of facilitated glucose transporter, member 1 (*SLC2A1*, previously known as *GLUT1*), facilitated glucose transporter, member 4 (*SLC2A4*, previously known as GLUT4), peroxisome proliferator-activated receptor γ (*PPARG*), peroxisome proliferator-activated receptor γ co-activator 1α (*PPARGC1A*), 11β-hydroxysteroid dehydrogenase-1 (*HSD11B1*), *TNF*, adiponectin (*ADIPOQ*), *IL6* and the macrophage marker *CD68* were measured using real-time PCR. *Results:* Basal expression of 'insulin-sensitivity genes' *SLC2A4* and *ADIPOQ* was lower while that of 'insulin-resistance genes', *HSD11B1* and *IL6* was significantly higher in the insulin-resistant than in the insulin-sensitive group. Insulin significantly increased expression of 'insulin-sensitivity genes' *SLC2A4*, *PPARG*, *PPARGC1A* and *ADIPOQ* in the insulin-sensitive group, while only expression of *PPARG* and *PPARGC1A* was increased in the insulin-resistant group. The expression of 'insulin-resistance genes' *HSD11B1* and *IL6* was increased by insulin in the insulin-resistant group, but insulin failed to increase *HSD11B1* expression in the insulin-sensitive group. At 6 h, expression of *HSD11B1*, *TNF* and *IL6* was significantly higher in the insulin-resistant than in the insulin-sensitive group. *IL6* expression increased significantly more in response to insulin in the insulin-resistant than in the insulin-sensitive group. *CD68* was overexpressed in the insulin-resistant as compared with the insulin-sensitive group at both 0 and 6 h. *Conclusions/interpretation:* These data suggest that genes adversely affecting insulin sensitivity hyperrespond to insulin, while genes enhancing insulin sensitivity hyporespond to insulin in insulin-resistant human adipose tissue in vivo.

Keywords Adipocytokines · Adiponectin · Cortisol · Interleukin · Macrophages · PGC-1 · TNF

Abbreviations *ACTB*: gene encoding β-actin · *ADIPOQ*: gene encoding adiponectin · *HSD11B1*: gene encoding 11β-hydroxysteroid dehydrogenase-1 · PPARG: peroxisome proliferator-activated receptor γ · PPARGC1A: peroxisome proliferator-activated receptor γ co-activator α · SLC2A1: facilitated glucose transporter, member 1 · SLC2A4: facilitated glucose transporter, member 4 · *TBP*: gene encoding TATA-box binding protein

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Introduction

Low-grade systemic inflammation and insulin resistance frequently coexist. Serum concentrations of cytokines, such as IL6 [1–6] and in some studies TNF [6, 7] correlate with markers of insulin resistance. Adipose tissue is one site of inflammation in insulin-resistant conditions. The number of macrophages is increased in both obese and lipoatrophic insulin-resistant subjects [8–10], and the expression of genes encoding IL6 [2, 6] and TNF [2, 6, 7] is increased in human

adipose tissue. IL6 impairs insulin signalling in 3T3-L1 adipocytes in vitro [11], and adipose tissue IL6 content correlates inversely with insulin action in vivo in humans [12]. The increased *TNF* expression in adipose tissue was recently suggested to originate exclusively from macrophages [8].

Insulin-resistant adipose tissue also overexpresses other genes that possibly contribute to insulin resistance, e.g. the gene encoding the enzyme that converts cortisone to cortisol, 11β-hydroxysteroid dehydrogenase-1 (*HSD11B1*), which is expressed in both adipocytes and macrophages [13, 14]. Overexpression of this enzyme in mice results in insulin resistance and visceral obesity [15]. In addition to these 'insulin-resistance' genes, another set of genes ('insulin-sensitivity genes') are underexpressed in insulin-resistant adipose tissue. These include the gene encoding adiponectin (*ADIPOQ*), which is deficient in both serum and adipose tissue of obese [16–18] and lipoatrophic [19] subjects, as well as the genes encoding the insulin-sensitive facilitated glucose transporter, member 4 (*SLC2A4*, previously known as GLUT4) [20], the adipogenic transcription factor peroxisome proliferator-activated receptor-γ (*PPARG*) [21, 22] and its coactivator 1α (*PPARGC1A*) [23, 24].

Regarding acute regulation of 'insulin-resistance' genes, insulin has been found to increase *IL6* expression in vitro in human adipocytes [25] and in 3T3-L1 cells [26], and transiently in a study in vivo in human adipose tissue [27]. Data on *TNF* expression are inconsistent, with one study reporting a transient increase in *TNF* mRNA by insulin in vivo with no change in serum TNF [27], while another in vivo study [28] and one in vitro study using human adipose tissue [29] found no effect of insulin on *TNF* expression. Insulin has been reported to decrease *HSD11B1* expression in vitro [13], and not to change expression during a 3-h insulin infusion in human adipose tissue [28]. Insulin acutely increases *SLC2A4* [30, 31] and *PPARG* [32] expression, but there are no in vivo data on acute regulation of *PPARGC1A* or *ADIPOQ* in vivo in human adipose tissue.

No studies to date have compared the acute effects of insulin on 'insulin-resistance' and '-sensitivity' genes in insulin-resistant and -sensitive subjects. In the present study, we compared expression of selected 'insulin-resistance' genes (*IL6*, *TNF*, *HSD11B1*) and 'insulin-sensitivity' genes (*SLC2A4*, *PPARG*, *PPARGC1A*, *ADIPOQ*) in subcutaneous adipose tissue biopsies of insulin-sensitive and insulin-resistant subjects, and compared responses of these genes to acute hyperinsulinaemia by repeating the biopsies after 3 and 6 h of insulin infusion. We also determined whether expression of the macrophage marker *CD68* is increased in the insulin-resistant vs -sensitive subjects, and whether its expression is regulated by insulin.

Subjects, materials and methods

Subjects and study designs A total of 21 non-diabetic apparently healthy Caucasian women were recruited on the basis of the following inclusion criteria: (1) age 18–60 years, and (2) no known acute or chronic disease other than obesity based on history and physical examination and standard laboratory tests (blood counts, serum creatinine, thyroid-stimulating hormone, electrolyte concentrations and electrocardiogram). Other exclusion criteria included pregnancy and treatment with drugs that may alter glucose tolerance. In each subject, whole-body insulin sensitivity was measured using the euglycaemic insulin clamp technique (insulin infusion rate 1 mU kg^{-1} min^{-1} for 6 h) and needle biopsies of adipose tissue were taken before and after 3 and 6 h of hyperinsulinaemia. The women were divided into insulin-sensitive (n=11) and insulin-resistant (n=10) groups, on the basis of their median rate of whole-body insulin sensitivity.

The nature and potential risks of the study were explained to all subjects prior to obtaining their written informed consent. The study was carried out in accordance with the principles of the Declaration of Helsinki. The protocol was approved by the ethics committee of Helsinki University Central Hospital.

Whole-body insulin sensitivity Whole-body insulin sensitivity was measured using the insulin clamp technique [33]. The study was begun at 07:30 hours after an overnight fast. Two 18-gauge catheters (Venflon; Viggo-Spectramed, Helsingborg, Sweden) were inserted, one in an antecubital vein for infusion of insulin and glucose, and another retrogradely in a heated hand vein to obtain arterialised venous blood for measurement of glucose concentrations every 5 min and serum free insulin concentration every 30 min. Regular human insulin (Insulin Actrapid; Novo Nordisk, Denmark) was infused in a primed-continuous fashion. The rate of the continuous insulin infusion was 1 mU kg^{-1} min^{-1} for 6 h. Normoglycaemia was maintained by adjusting the rate of a 20% glucose infusion based on plasma glucose measurements from arterialised venous blood every 5 min. Whole-body insulin sensitivity was determined from the glucose infusion rate required to maintain normoglycaemia between 30 and 360 min [33].

Adipose tissue biopsy and total RNA cDNA preparation A needle aspiration biopsy of abdominal subcutaneous fat was taken under local intracutaneous anaesthesia at baseline and after 3 and 6 h of hyperinsulinaemia [34]. Each biopsy sample was taken from different locations in the left, middle and right lower abdominal region. The sample was immediately frozen and stored in liquid nitrogen until analysis. Frozen tissue samples (50–150 mg) were homogenised in 2 ml RNA STAT-60 (Tel-Test, Friendswood, TX, USA) and total RNA isolated according to the manufacturer's instructions. After DNase treatment (RNase-free DNase Set; Qiagen, Hilden, Germany) RNA was purified using the RNeasy Minikit (Qiagen). RNA concentrations were measured using the RiboGreen fluorescent nucleic acid stain (RNA Quantification Kit; Molecular Probes, Eugene, OR, USA). The quality of RNA was checked by agarose gel electrophoresis. Isolated RNA was stored at −80°C until quantification of target mRNAs. A total of 0.1 μg RNA was transcribed into cDNA using Moloney murine leukaemia virus reverse transcriptase (Life Technologies, Paisley, UK) and oligo $(dT)_{12–18}$ primers.

Quantification of mRNA concentrations Quantification of the gene encoding β-actin (*ACTB*), *PPARG* and *ADIPOQ* mRNA was performed in Helsinki (E.K.) by real-time PCR using LightCycler technology (Roche Diagnostics, Mannheim, Germany). An aliquot of 2 μl 1:10 diluted cDNA was brought to a final volume of 20 μl, which contained 3 mmol/l magnesium chloride, 2 μl LightCycler-FastStart DNA SYBR Green I Mix (Roche Diagnostics) and 0.5 μmol/l primers. After the initial activation of the DNA polymerase at 95°C for 10 min, the amplification conditions were as follows: 40 cycles consisting of denaturation at 95°C for 15 s, annealing for 5 s at 57°C (*ACTB*), 56°C (*PPARG*) and 58°C (*ADIPOQ*) and extension at 72°C. The extension times (s) were calculated from the amplicon size (bp/25). Fluorescent data were acquired at the end of each extension phase. After amplification, a melting curve analysis from 65°C to 95°C with a heating rate of 0.1°C/s with a continuous fluorescence acquisition was made. The primers for *PPARG*, *ADIPOQ* and *ACTB* have been described [35]. A standard curve for *PPARG* was created using purified cloned plasmid cDNA (QIAquick PCR Purification Kit; Qiagen). For human *ACTB* and *ADIPOQ* expression, standard curves were created from a specific PCR product. To account for differences in RNA loading, *PPARG* and *ADIPOQ* were expressed relative to *ACTB*.

The mRNA expression levels of facilitated glucose transporter, member 1 (*SLC2A1*, previously known as *GLUT1*), *SLC2A4, PPARGC1A, TNF,HSD11B1* and the gene encoding TATA-box binding protein (*TBP*) were measured in Stockholm (K.K., M.K., R.M.F.) using TaqMan real-time PCR according to the manufacturer's protocol using an ABI PRISM 7000 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA, USA). cDNA synthesised from 15 ng total RNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems). Primer and probe sets for *SLC2A1, SLC2A4, PPARGC1A* and *HSD11B1* were designed using the manufacturer's software and sequences available in GeneBank, and the sequences have been published previously [35, 36]. Differences in the loading of cDNA were adjusted for by expressing results relative to *TBP*. Expression levels were quantified in arbitrary units by generating a six-point serial standard curve. After these analyses were performed, obese subjects were reported to have an excess of macrophages in adipose tissue [8, 9], and IL6 was shown to cause insulin resistance in adipocytes [11]. Therefore *CD68* and *IL6* were quantified later in remaining 0- and 6-h samples and expressed relative to TBP. *TNF,CD68, IL6* and *TBP* were measured using Pre-Developed TaqMan Assay Reagents (assay numbers Hs00174128_m1, Hs00154355_m1, Hs0 0174131_m1 and Hs99999910_m1, respectively; Applied Biosystems). The specificity of each primer and probe set was confirmed by visualisation of a single PCR product by agarose gel electrophoresis.

Other measurements Blood samples were taken after an overnight fast for measurement of plasma glucose, serum insulin and C-peptide, serum triglyceride and total and HDL cholesterol concentrations. The percentage (%) of body fat was determined by using bioelectrical impedance analysis (BioElectrical Impedance Analyzer System Model #BIA-101A; RJL Systems, Detroit, MI, USA) [37]. Waist circumference was measured midway between spina iliaca superior and the lower rib margin, and hip circumference at the level of the greater trochanters [38].

Analytical procedures Plasma glucose concentrations were measured in duplicate with the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Fullerton, CA, USA) [39]. Serum free insulin concentrations were measured using the Auto-DELFIA kit from Wallac (Turku, Finland) and C-peptide concentrations by RIA [40]. HbA_1c was measured by HPLC using the fully automated Glycosylated Hemoglobin Analyzer System (BioRad, Richmond, CA, USA) [41]. Serum total cholesterol, HDL cholesterol and triglyceride concentrations were measured with enzymatic kits from Roche Diagnostics using an autoanalyser (Roche Diagnostics Hitachi 917; Hitachi, Tokyo, Japan). LDL cholesterol concentration was calculated using the formula of Friedewald [42]. Serum adiponectin concentrations were measured using an ELISA kit from B-Bridge International (San Jose, CA, USA).

Table 1 Physical and biochemical characteristics of the study subjects divided into insulin-sensitive and -resistant groups on the basis of their median whole-body insulin sensitivity

	Insulin-sensitive	Insulin-resistant	*p* value
Number	11	10	–
Age (years)	32±3	40±3	NS
Body weight (kg)	69±4	90±4	<0.01
BMI (kg/m^2)	24.7±1.1	32.7±1.8	<0.001
Whole-body fat (%)	28±2	36±1	<0.001
Fat mass (kg)	20±2	35±4	<0.01
Waist-to-hip-ratio	0.86±0.01	0.91±0.01	<0.01
Fasting plasma glucose (mmol/l)	5.1±0.1	5.6±0.2	<0.01
Fasting serum insulin (mU/l)	3±1	10±1	<0.001
Fasting serum C-peptide (nmol/l)	0.4±0.1	0.8±0.1	<0.001
Fasting serum LDL cholesterol (mmol/l)	2.2±0.1	3.1±0.1	<0.01
Fasting serum triglycerides (mmol/l)	0.8±0.1	1.4±0.2	<0.01
Fasting serum HDL cholesterol (mmol/l)	1.4±0.1	1.3±0.1	<0.001
Fasting serum adiponectin (mg/l)	18±2	12±1	<0.01

Data are means±SEM

Statistical analyses All parameters were analysed using non-parametric methods. Insulin-sensitive and -resistant groups were compared using the Mann–Whitney test. Effects of insulin were analysed using Friedman's test followed by Dunn's post hoc test to compare single measurements. Correlations were calculated using Spearman's rank correlation coefficient. $p>0.05$ was considered statistically significant. The calculations were performed using SPSS 11.0 for Windows (SPSS, Chicago, IL, USA). All data are shown as means±SEM.

Results

Clinical characteristics Characteristics of the groups are given in Table 1. The insulin-resistant group was more obese than the insulin-sensitive group. Markers of insulin resistance, including serum fasting insulin, C-peptide and triglyceride concentrations were higher and HDL cholesterol and adiponectin concentrations were lower in the insulin-resistant than in the insulin-sensitive group.

Table 2 The relationship (Spearman's *r*) between gene expression in adipose tissue and whole-body insulin sensitivity

Gene	0 h	3 h	6 h
SLC2A1/TBP	−0.08	−0.29	0.04
Insulin-sensitivity genes			
SLC2A4/TBP	0.44#	0.66**	0.73***
PPARG/ACTB	0.14	−0.37	−0.14
PPARGC1A/TBP	0.19	0.55*	0.34
ADIPOQ/ACTB	0.48*	−0.16	−0.24
Insulin-resistance genes			
IL6/TBP	−0.48*	ND	−0.71***
TNF/TBP	−0.44*	−0.53*	−0.46*
HSD11B1/TBP	−0.55*	−0.74***	−0.82***
Macrophage marker			
CD 68/TBP	−0.66**	ND	−0.68***

ND not determined
*$p<0.05$
**$p<0.01$
***$p<0.001$
#$p=0.06$

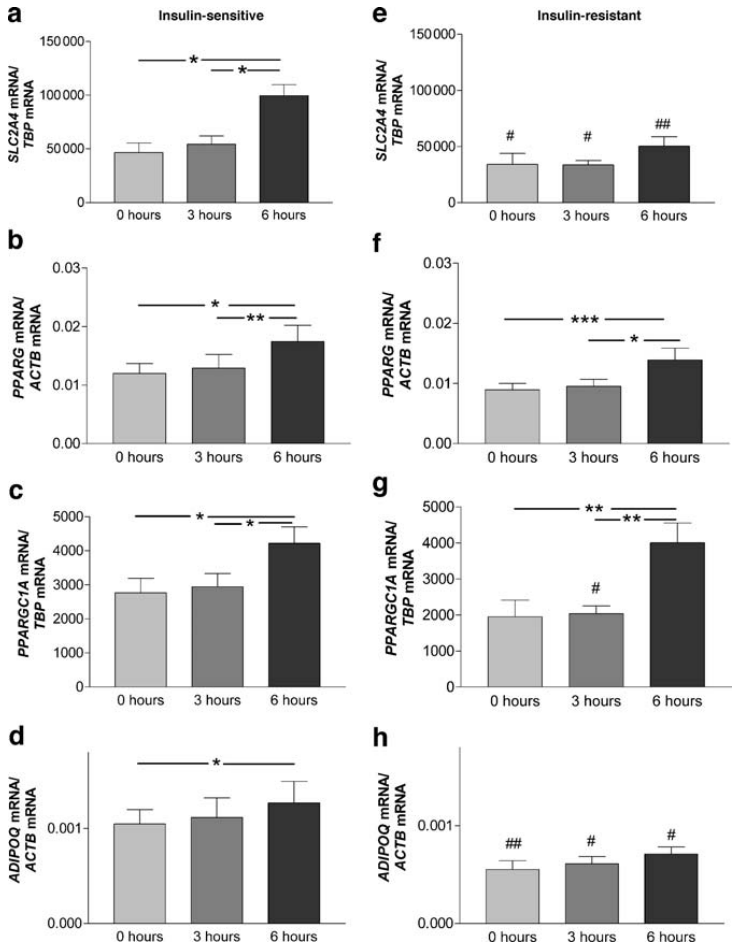

Fig. 1 The expression of 'insulin-sensitivity genes' *SLC2A4*, *PPARG*, *PPARGC1A* and *ADIPOQ* in (**a**–**d**) insulin-sensitive (n=11) and (**e**–**h**) insulin-resistant (n=10) subjects at 0, 3 and 6 h during euglycaemic hyperinsulinaemia (rate of continuous insulin infusion 1 mU kg^{-1} min^{-1}). *$p<0.05$, **$p<0.01$, ***$p<0.001$ for change between time points; #$p<0.05$, ##$p<0.01$ for difference between groups

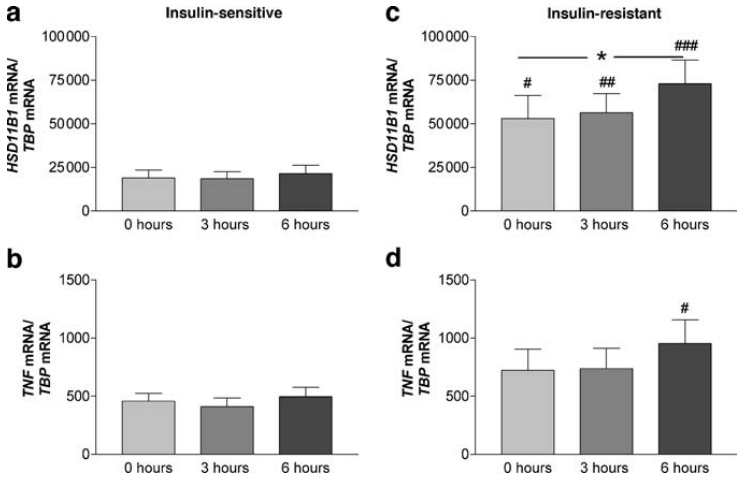

Fig. 2 The expression of 'insulin-resistance genes' *HSD11B1* and *TNF* in (**a, b**) insulin-sensitive (n=11) and (**c, d**) insulin-resistant (n=10) subjects at 0, 3 and 6 h during euglycaemic hyperinsulinaemia (rate of continuous insulin infusion 1 mU kg^{-1} min^{-1}). *p<0.05 for change between time points; #p<0.05, ##p<0.01, ###p<0.001 for difference between groups

During the insulin infusion, serum insulin concentrations were similar in insulin-sensitive and -resistant groups (69±4 vs 76±4 mU/l, respectively; NS). By definition, whole-body insulin sensitivity was 107% higher in the insulin-sensitive than the insulin-resistant group (8.7±0.4 vs 4.2±0.3 mg kg^{-1} min^{-1}, p<0.0001).

Expression of genes encoding SLC2A1, SLC2A4, PPARG, PPARGC1A and ADIPOQ in adipose tissue Before the start of the insulin infusion, the mRNA concentrations of *SLC2A1* (1033±543 vs 722±251, insulin-sensitive vs -resistant; NS), *PPARG* and *PPARGC1A* (Fig. 1) were comparable between the two groups. *SLC2A4* gene expression was lower in the insulin-resistant than the insulin-sensitive group at baseline (p<0.05). The mRNA concentrations of *SLC2A1* and the housekeeping genes (*ACTB* and *TBP*) were comparable between the groups and remained unchanged during insulin infusion in both groups (data not shown). During the insulin infusion, *SLC2A4* mRNA concentrations increased 2.2-fold in the insulin-sensitive group, whereas expression of *SLC2A4* remained unchanged in the insulin-resistant group (Fig. 1). At 6 h, *SLC2A4* expression was significantly higher in the insulin-sensitive than the insulin-resistant group. In univariate correlation analysis (all subjects analysed as one group), *SLC2A4* mRNA correlated with whole-body insulin sensitivity at 3 h (Spearman's r=0.66, p<0.01) and 6 h (r=0.73, p<0.001) and almost significantly at 0 h (Table 2). The expression of *PPARG* and *PPARGC1A* increased significantly during insulin infusion in both groups with no differences between the groups (Fig. 1). *PPARGC1A* and *SLC2A4* mRNA concentrations correlated with each other at 0 h (r=0.66, p<0.01) and 6 h (r=0.38, p<0.05). The changes in gene expression between 0 and 6 h of insulin infusion were confirmed in independent assays for the quantification of *SLC2A4*, *PPARG* and *PPARGC1A* (data not shown).

ADIPOQ gene expression at all time points was significantly higher in the insulin-sensitive than the insulin-resistant group (Fig. 1). *ADIPOQ* mRNA concentrations increased significantly in the former but not in the latter group (Fig. 1). Serum adiponectin concentrations were significantly higher in the insulin-sensitive than the insulin-resistant group before (Table 1) and during insulin infusion at 3 h (16±2 vs 11±1 mg/l, respectively; p<0.01) and 6 h (16±2 vs 11±1 mg/l, p<0.01).

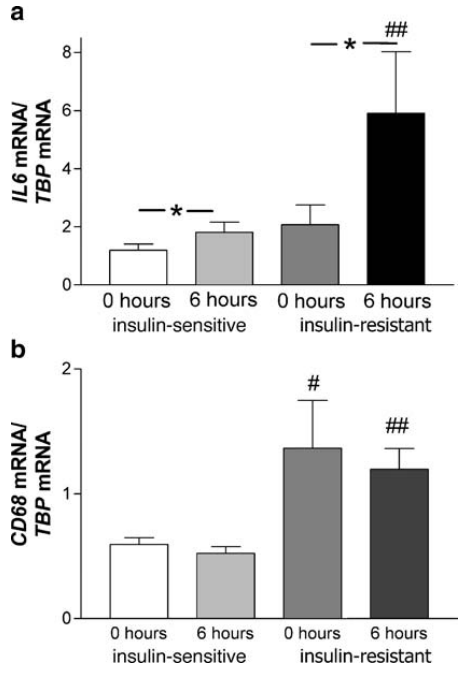

Fig. 3 The expression of (**a**) *IL6* and (**b**) the macrophage marker *CD68* in insulin-sensitive and insulin-resistant subjects at 0 and 6 h during euglycaemic hyperinsulinaemia (rate of continuous insulin infusion 1 mU kg^{-1} min^{-1}). *p<0.05 for change between time points; #p<0.05, ##p<0.01 for difference between groups

HSD11B1, TNF and *IL6* Before insulin infusion, *HSD11B1* mRNA concentrations were 2.4-fold higher in the insulin-resistant than the insulin-sensitive group (Fig. 2). Insulin further increased *HSD11B1* mRNA concentrations in the former group, while there were no changes in the insulin-sensitive group. *TNF* mRNA concentrations tended to be higher in the insulin-resistant than the insulin-sensitive group at all time points with a significant difference at 6 h (Fig. 2). Insulin did not change *TNF* concentrations significantly. *IL6* gene expression increased by insulin in both groups and expression was significantly higher in the insulin-resistant than in the insulin-sensitive group at 6 h (Fig. 3). Also, the increase in *IL6* gene expression by insulin was significantly higher in the insulin-resistant than the insulin-sensitive group ($p<0.02$).

Expression of macrophage marker CD68 Before and during the insulin infusion, *CD68* mRNA concentrations were significantly higher in the insulin-resistant than the insulin-sensitive group (Fig. 3).

Discussion

The present data are the first to compare the responses of genes thought to counteract insulin action ('insulin-resistance genes') and enhance insulin action ('insulin-sensitivity genes') to insulin in vivo in adipose tissue. The study was performed in apparently healthy subjects (except for obesity) who were arbitrarily divided into less insulin-sensitive (insulin-resistant group) and more insulin-sensitive (insulin-sensitive group) based on their median whole-body insulin sensitivity. The results show that insulin resistance is not only characterised by failure of insulin to normally increase expression of 'insulin-sensitivity genes' (*SLC2A4*, *ADIPOQ*) but also by hyperresponsiveness of 'insulin-resistance genes' (*IL6*, *HSD11B1*, *TNF*) to insulin.

The multitude of genes involved in regulating insulin action and the discovery that adipose tissue of obese subjects contains an excess of macrophages [8], which also express insulin-action genes, makes interpretation of the present data complex. Because of the need for repeated biopsies, it was unfeasible to take enough adipose tissue to allow separation of macrophages and adipose cells, and therefore we were unable to allocate the observed changes in gene expression to a given cell type. Theoretically, this implies that any of the following scenarios might have taken place. Firstly, genes hyperresponding to insulin could reside in a different cell type than those hyporesponding to insulin. For example, the hyperresponse to insulin of *IL6*, which is expressed both in macrophages and in adipocytes, could have occurred in macrophages and the hyporesponse of adiponectin in adipocytes, which is the only cell type expressing adiponectin [43]. Secondly, both the hyper- and hyporesponse could have occurred in the same cell type but the increased basal or insulin-stimulated expression of 'insulin-resistance genes' in the insulin-resistant group could have modulated the response to insulin. Thirdly, a combination of both mechanisms might have been involved. These possibilities will be further discussed below in conjunction with the individual genes.

We divided the present study subjects on the basis of their whole-body insulin sensitivity. As expected the insulin-resistant subjects were more obese and had more whole-body fat than the insulin-sensitive subjects. We cannot therefore distinguish between effects of obesity as compared with insulin sensitivity on gene expression in adipose tissue. Adipocyte cell size is increased in insulin-resistant obese subjects and may influence adipose gene expression [44]. There are abundant, albeit controversial, data on differences in gene expression between different adipose tissue depots [45]. Repeated sampling of intra-abdominal fat was not ethically justifiable in healthy subjects.

Several previous studies have compared expression of one or several of the genes in the basal state, i.e. after an overnight fast, between non-obese insulin-sensitive and obese insulin-resistant subjects. These include reports of decreased expression of the 'insulin-sensitivity genes' *SLC2A4* [20, 46, 47], *PPARGC1A* [23] and *ADIPOQ* [16, 48]. These findings were confirmed in the present study. Deletion or overexpression of each of these genes has been shown to modulate insulin action in mouse models [21, 49–52]. Data are inconsistent regarding *PPARG*, with reports of decreased [53, 54] or unchanged [55, 56] expression of the gene encoding PPARG1, and decreased [57], unchanged [53, 55] or increased [54, 56] expression of the gene encoding PPARG2 in obese as compared with lean subjects. The relative abundance of the mRNAs of *PPARG1* and *PPARG2* is also controversial [32, 55–57]. In the present study, *PPARG* expression was comparable between the insulin-resistant and the insulin-sensitive group (Fig. 1). Regarding expression of the 'insulin-resistance genes', we confirm reports of increased basal expression of *HSD11B1* [58, 59]. In addition, at 6 h of insulin infusion gene expression of *TNF* and *IL6* was greater in the insulin-resistant than in the insulin-sensitive group (Figs. 2 and 3).

Regarding acute regulation of gene expression by insulin, the present data are, to the best of our knowledge, novel in comparing acute changes in gene expression in insulin-sensitive and -resistant subjects and in demonstrating that insulin increases mRNA concentrations of *HSD11B1* and *PPARGC1A* in human adipose tissue in vivo. Previous studies have shown that insulin increases *SLC2A4* expression in skeletal muscle [30, 60], and that this effect is blunted in skeletal muscle in insulin-resistant as compared with insulin-sensitive subjects [30, 60]. This was confirmed in adipose tissue in the present study. PPARGC1A is a transcriptional coactivator, which induces gene expression of *SLC2A4* in skeletal muscle [61]. It is absent from white adipose tissue in mice but is expressed in human adipose tissue [23]. We confirm the presence of *PPARGC1A* in human adipose tissue [23] and extend previous findings by demonstrating that insulin acutely increases *PPARGC1A* expression in vivo. *PPARGC1A* and *SLC2A4* expression was significantly correlated, which is in keeping with data showing that overexpression of *PPARGC1A* in skeletal muscle increases *SLC2A4* content and insulin sensitivity

[24]. Overexpression of *PPARGC1A* increases energy expenditure by stimulating thermogenesis in brown fat in mice [62]. This increases the need for fuels such as glucose. Increases in gluconeogenesis in the liver and glucose utilisation in peripheral tissues by PPARGC1A could help to maintain energy supply, even if regulated in a tissue-specific fashion by insulin. Insulin has been shown to acutely (i.e. within 3 h) increase expression of *PPARG*, but the response was similar in lean and obese subjects and in type 2 diabetic patients [32]. In the present study, we also found that *PPARG* gene expression responded similarly to insulin in both groups studied (Fig. 1).

Regarding 'insulin-resistance genes', *HSD11B1* was higher at all time points in the insulin-resistant as compared with the insulin-sensitive group, and increased in response to insulin in the former but not in the latter group (Fig. 2). Data regarding insulin regulation of *HSD11B1* gene expression and activity are not unequivocal. In preadipocytes, insulin attenuates HSD11B1 activity but synergises with glucocorticoids to stimulate adipocyte differentiation that is associated with induction of HSD11B1 activity [63]. In vivo, consistent with the present data at the 3 h time point, 3 h of hyperinsulinaemia has been reported not to change *HSD11B1* expression in type 2 diabetic or healthy men [28]. *HSD11B1* is a gene that is abundantly expressed in macrophages in addition to adipocytes [14]. Therefore the increase in *HSD11B1* in the insulin-resistant group could also be due to insulin action in macrophages, although this possibility remains hypothetical. Like *HSD11B1*, *IL6* was increased significantly more by insulin in the insulin-resistant than the insulin-sensitive subjects (Fig. 3). This most probably occurred in non-adipose cells, since isolated adipocytes account for only 10% of total IL6 release from human adipose tissue [4]. *IL6* may antagonise insulin action [1] by several mechanisms. It has been shown to down-regulate *SLC2A4*, *PPARG* and *ADIPOQ* expression in 3T3-L1 adipocytes [11, 64, 65], and increase HSD11B1 activity in primary cultures of adipose stromal cells [66]. The interstitial IL6 concentration in human adipose tissue is ~100 times higher than that in plasma [67] and IL6, in contrast to TNF, is released from adipose tissue systemically [3]. The high local concentration of IL6 and the existence of all components important for IL6 signalling in human fat cells suggest that IL6 originating from non-fat cells in part induces insulin resistance in a paracrine fashion [11].

TNF expression after 6 h of insulin infusion was higher in insulin-resistant than in insulin-sensitive subjects. Two recent studies have found virtually all TNF to be derived from macrophages [9] or non-fat cells [68] in human adipose tissue. In a study examining the direct effect of insulin on macrophage gene expression using an array technique, insulin stimulated *TNF* the most among all genes in the analysis [69]. In an earlier study, lipopolysaccharide was found to increase TNF production more robustly from human whole-adipose tissue than from isolated adipocytes [29]. In the latter study, insulin had no effect on TNFproduction. In a study that also used the insulin clamp technique and measured *TNF* expression in a group of normal subjects, insulin was found to increase *TNF* expression in adipose tissue transiently after 2 h [27]. In another study, 4 h of in vivo hyperinsulinaemia had no effect on subcutaneous adipose tissue *TNF* mRNA concentration in lean or obese subjects [28]. Our data suggest that the response of *TNF* to insulin is exaggerated in insulin-resistant subjects, although the increase by insulin failed to reach statistical significance. Whether this is because an increased number of macrophages (vide infra) hyperrespond to insulin remains to be determined.

In the study reporting macrophage accumulation in adipose tissue in obesity, immunohistochemical detection and quantification of *CD68*-expressing cells in subcutaneous adipose tissue showed that average adipocyte size and BMI were strong predictors of *CD68*-expressing cells [9]. The increase in the present study of *CD68* expression in insulin-resistant obese subjects therefore most probably reflects an increase in the number of macrophages in adipose tissue.

ADIPOQ mRNA concentration increased significantly by 6 h in the insulin-sensitive but not the insulin-resistant group (Fig. 1). *ADIPOQ* expression has been studied in two previous in vivo studies, which found no change after 2.5 h [48] and 3 h [28] of insulin infusion, consistent with the present data after 3 h. In vitro, in 3T3-L1 adipocytes, insulin has been reported both to increase [70] and decrease [71] adiponectin expression. In human adipocytes, insulin has been reported to increase adiponectin secretion from omental but not subcutaneous adipocytes [72].

We conclude that acute insulin regulation of gene expression in insulin-resistant adipose tissue is characterised not only by hyporesponsiveness of insulin-sensitivity genes such as *SLC2A4* to insulin but also by hyperresponsiveness of insulin-resistance genes (*IL6*, *TNF*, *HSD11B1*). Although attempts made to date in humans using anti-inflammatory approaches such as TNF antagonists to ameliorate insulin resistance have been unsuccessful, the present study supports continued development of such therapies.

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Insulin regulation of MCP-1 in human adipose tissue of obese and lean women

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Westerbacka J, Cornér A, Kolak M, Makkonen J, Turpeinen U, Hamsten A, Fisher RM, Yki-Järvinen H. Insulin regulation of MCP-1 in human adipose tissue of obese and lean women. *Am J Physiol Endocrinol Metab* 294: E841–E845, 2008. First published 12 February 2008; doi:10.1152/ajpendo.00653.2006.—CCL2 (MCP-1, monocyte chemoattractant protein 1) and CCL3 (MIP-1α, macrophage inflammatory protein 1α) are required for macrophage infiltration in adipose tissue. Insulin increases CCL2 expression in adipose tissue and in serum more in insulin-resistant obese than in insulin-sensitive lean mice, but whether this is true in humans is unknown. We compared basal expression and insulin regulation of CCL2 and CCL3 in adipose tissue and MCP-1 and MIP-1α in serum between insulin-resistant and insulin-sensitive human subjects. Subcutaneous adipose tissue biopsies and blood samples were obtained before and at the end of 6 h of in vivo euglycemic hyperinsulinemia (maintained by the insulin clamp technique) in 11 lean insulin-sensitive and 10 obese insulin-resistant women, and before and after a 6-h saline infusion in 8 women. Adipose tissue mRNA concentrations of monocyte/macrophage markers CD68, EMR1, ITGAM, ADAM8, chemokines CCL2 and CCL3, and housekeeping gene ribosomal protein large P0 (RPLP0) were measured by means of real-time PCR at baseline. In addition, mRNA concentrations of CCL2, CCL3, and RPLP0 were measured after insulin infusion. Levels of MCP-1 and MIP-1α were determined in serum, and protein concentration of MCP-1 was determined in adipose tissue at baseline and after insulin infusion. Basally, expression of the macrophage markers CD68 and EMR1 were increased in adipose tissue of insulin-resistant subjects. Insulin increased MCP-1 gene and protein expression significantly more in the insulin-resistant than in the insulin-sensitive subjects. Basally expression of CCL2 and CCL3 and expression of macrophage markers CD68 and ITGAM were significantly correlated. In serum, MCP-1 decreased significantly in insulin-sensitive but not insulin-resistant subjects. MIP-1α was undetectable in serum. Insulin regulation of CCL2 differs between insulin-sensitive and -resistant subjects in a direction that could exacerbate adipose tissue inflammation.

adipocytes; adipokines; insulin sensitivity; monocyte chemoattractant protein

MCP-1 PLAYS A KEY ROLE in recruitment of monocytes but not neutrophils or eosinophils to sites of injury (6, 26). It also induces insulin resistance in adipocytes via downregulation of genes such as SLC2A4 (the gene encoding GLUT-4), lipoprotein lipase, and peroxisome proliferator-activated receptor-γ (16). Recently, it was demonstrated that deletion of the CCR2 receptor for monocyte chemoattractant protein (MCP)-1 in obese mice strains matched for adiposity reduced macrophage content and the inflammatory profile of adipose tissue, increased adiponectin expression, ameliorated hepatic steatosis, and improved systemic glucose homeostasis and insulin sensitivity (21). In mice with established obesity, short-term treatment with a pharmacological antagonist of MCP-1 lowered macrophage content of adipose tissue and improved insulin sensitivity without significantly altering body mass or improving hepatic steatosis (21).

In murine adipocytes in vitro and ob/ob mice in vivo, insulin increases expression and secretion of MCP-1 (16). In the latter in vivo experiment, the response to insulin was exaggerated in obese insulin-resistant compared with lean mice (16). Thus, as previously described for plasminogen activator inhibitor (PAI)-1 (15) and sterol regulatory element binding protein (SREBP)-1c (17) in mice, MCP-1 may retain its sensitivity to insulin or even hyperrespond to insulin in insulin-resistant states. Hyperinsulinemia might under such conditions accelerate monocyte recruitment and worsen insulin resistance (16). These data may or may not be relevant to humans, since insulin has been reported to decrease serum MCP-1 levels in humans (5), and since catheterization studies of human subcutaneous tissues have suggested that MCP-1 is not released systematically (4). There are no studies comparing the response of CCL2 expression or of its levels in serum to insulin between insulin-sensitive and insulin-resistant human subjects.

In the present study, we examined how acute in vivo hyperinsulinemia in human insulin-resistant compared with insulin-sensitive subjects regulates expression of the chemokines CCL2 and CCL3 and MCP-1 protein concentration in adipose tissue. We also determined whether expression of multiple macrophage markers basally is correlated with chemokine expression. The latter genes included CD68 and EMR1 (epidermal growth factor module-containing mucin-like hormone receptor 1), and ADAM8 (a disintegrin and metallopeptidase domain 8), which are macrophage-specific markers, and ITGAM (integrin, alpha M), which is an integrin found in monocytes, macrophages, neutrophils, and NK cells.

MATERIALS AND METHODS

Subjects and study designs. A total of 21 nondiabetic Caucasian women were recruited based on the following inclusion criteria: *1*) age 18–60 years; *2*) no known acute or chronic disease other than obesity based on history and physical examination and standard

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laboratory tests (blood counts, serum creatinine, thyroid stimulating hormone, electrolyte concentrations, and electrocardiogram); and *3*) body mass index (BMI) $<$ 40 kg/m2. Other exclusion criteria included treatment with drugs that may alter glucose tolerance or pregnancy. In each subject, whole-body insulin sensitivity was measured according to the euglycemic insulin clamp technique (insulin infusion rate 1 mU·kg$-$1·min$-$1 for 6 h), and needle biopsies of adipose tissue were taken before and after 6 h of hyperinsulinemia. The women were divided into lean insulin-sensitive (n = 11, age 32 $\pm$ 3 yrs, body wt 69 $\pm$ 4 kg, BMI 24.7 $\pm$ 1.1 kg/m2) and obese insulin-resistant (n = 10, age 40 $\pm$ 3 yrs, body wt 90 $\pm$ 4 kg, BMI 32.7 $\pm$ 1.8 kg/m2) based on their median rate of whole-body insulin sensitivity measured by the euglycemic insulin clamp technique (23). There were two postmenopausal women in both groups. In 8 women (age 36 $\pm$ 4 yrs, BMI 28.2 $\pm$ 2.9 kg/m2), CCL2 and CCL3 expressions were measured in adipose tissue before and at the end of a 6-h saline infusion.

The nature and potential risks of the study were explained to all subjects prior to obtaining their written informed consent. The study was carried out in accordance with the principles of the Declaration of Helsinki. The protocol was approved by the ethics committee of the Helsinki University Central Hospital.

Adipose tissue biopsy and total RNA cDNA preparation. Needle aspiration biopsies of abdominal subcutaneous fat were taken under local intracutaneous anesthesia at baseline and after 6 h of hyperinsulinemia from the left and right lower abdominal region (25). The samples were immediately frozen and stored in liquid nitrogen until analysis. Frozen tissue samples (50–150 mg) were homogenized in 2 ml RNA STAT-60 (Tel-Test, Friendswood, TX), and total RNA was isolated as previously described (19). RNA was stored at $-$80°C until quantification of target mRNAs. A total of 0.1 μg RNA was transcribed into cDNA via Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, UK) and oligo (dT)12–18 primers (19). Part of the fresh sample was used to isolate adipocytes through incubation with collagenase to measure adipocyte size with microscope (7).

Gene expression analyses. mRNA expression of CD68, EMR1, ITGAM, ADAM8, CCL2, CCL3, and ribosomal protein large P0 (RPLP0) was quantified by real-time PCR by means of the ABI 7000 Sequence Detection System instrument and software (Applied Biosystems). cDNA synthesized from 15 ng of total RNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and a gene-specific primer and probe mixture (predeveloped TaqMan Gene Expression Assays, Applied Biosystems) in a final volume of 15 μl. The assays used were: CD68, Hs00154355_m1; EMR1, Hs00173562_m1; ITGAM, Hs00355885_m1; ADAM8, Hs00174246_m1; CCL2, Hs00234140_m1; CCL3, Hs00234142_m1; and RPLP0, Hs99999902_m1. All samples were run in duplicate. Relative expression levels were determined by means of a 5-point serially diluted standard curve, generated from cDNA from human adipose tissue. Expression levels were expressed in arbitrary units and normalized relative to the housekeeping gene RPLP0 to compensate for differences in cDNA loading.

Measurement of MCP-1 protein expression in adipose tissue. A frozen sample (5 from the insulin-sensitive and 7 from the insulin-resistant group) of human subcutaneous adipose tissue (100–250 mg) was homogenized in lysis buffer. The homogenate was centrifuged for 30 min (+4°C, 14,000 rpm), and the supernatant was stored at $-$80°C until measurement of the MCP-1 concentration with the Human CCL2/MCP-1 Immunoassay kit (Quantikine; R&D Systems, Minneapolis, MN) and the Bio Assay Reader HTS 7000 Plus (Perkin Elmer, Norwalk, CT). Total protein was measured with the BC Assay-protein quantitation kit (Uptima Interchim, Montlucan, France).

Other measurements. Blood samples were taken after an overnight fast for measurement of plasma glucose, serum insulin, C-peptide, serum triglycerides, and total and HDL cholesterol concentrations, as described (23). Serum MCP-1 and macrophage inflammatory protein (MIP)-1α concentrations were measured with ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA).

Statistical analyses. All parameters were analyzed via nonparametric methods. Insulin-sensitive and insulin-resistant groups were compared via the Mann-Whitney test. Effects of insulin were analyzed via Friedman's test followed by Dunn's post hoc test to compare single measurements. Correlations were calculated via Spearman's rank correlation coefficient. A P value of less than 0.05 was considered statistically significant. The calculations were performed with SPSS 11.0 for Windows (SPSS, Chicago, IL). All data are shown as mean $\pm$ standard error of mean.

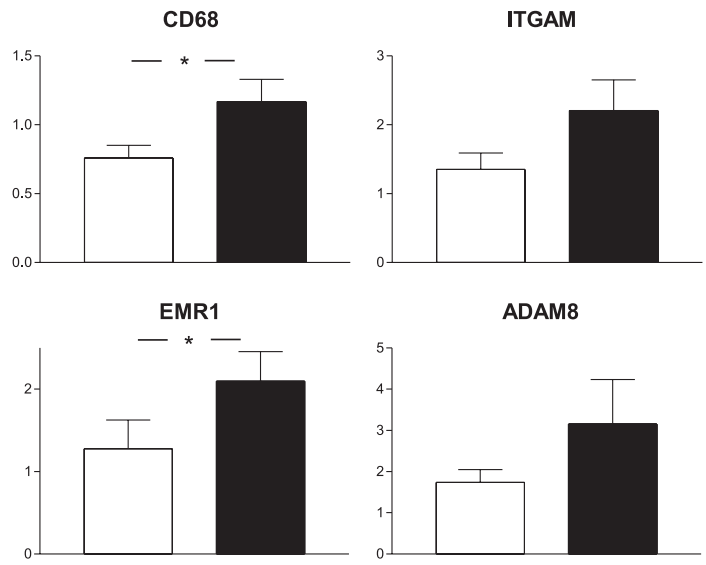

Fig. 1. Gene expressions of monocyte/macrophage markers CD68, ITGAM, EMR1 and ADAM8 relative to housekeeping gene (RPLP0) expression in adipose tissue in insulin-sensitive (IS, open bars) and insulin-resistant (IR, filled bars) subjects at baseline. *P $<$ 0.05 for IS vs. IR.

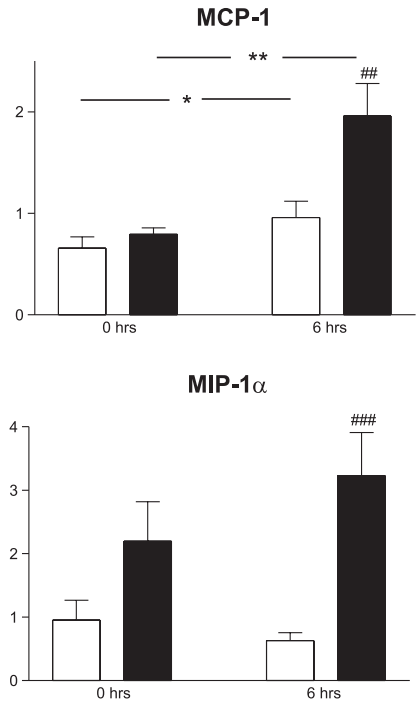

Fig. 2. The gene expressions of chemokines CCL2 (MCP-1, monocyte chemoattractant protein 1) and CCL3 (MIP-1α, macrophage inflammatory protein 1α) relative to housekeeping gene ribosomal protein large P0 (RPLP0) expression in adipose tissue in IS (open bars) and IR (filled bars) subjects at 0 and 6 h during euglycemic hyperinsulinemia (rate of continuous insulin infusion 1 mU·kg−1·min−1). *$P < 0.05$, **$P < 0.01$ for change between time points. ##$P < 0.01$, ###$P < 0.001$ for IS vs. IR.

RESULTS

The insulin-resistant group was more obese than the insulin-sensitive group (BMI 32.7 ± 1.8 vs. 24.7 ± 1.1, $P < 0.001$), and had higher insulin concentrations (10 ± 1 vs. 3 ± 1 mU/l, $P < 0.001$). During the insulin infusion, serum insulin concentrations were similar in insulin-sensitive and insulin-resistant groups (69 ± 4 vs. 76 ± 4 mU/l, insulin-sensitive vs. insulin-resistant, NS). Whole-body insulin sensitivity was 95% higher in the insulin-sensitive than in the insulin-resistant group (8.7 ± 0.4 vs. 4.2 ± 0.3 mg·kg−1·min−1, $P < 0.0001$). Other characteristics have been described in (23).

Gene expressions of CD68, EMR1, ITGAM, ADAM8, CCL2 (MCP-1), and CCL3 (MIP-1α), and MCP-1 protein concentration in adipose tissue. Before start of the insulin infusion, the mRNA concentrations of ITGAM, ADAM8, CCL2, and CCL3 were comparable between the insulin-sensitive and insulin-resistant groups, but the mRNA concentrations of CD68 and EMR1 were significantly higher in the insulin-resistant than in the insulin-sensitive group (Fig. 1). At baseline, CCL2 and CCL3 expressions correlated closely with those of CD68 ($r = 0.71$, $P < 0.001$ and $r = 0.81$, $P < 0.001$ for CCL2 and CCL3, respectively) and ITGAM ($r = 0.64$, $P < 0.01$ and $r = 0.78$, $P < 0.001$, respectively), and CCL3 correlated with EMR1 ($r = 0.55$, $P < 0.01$). CCL2 expression increased significantly more by insulin in the insulin-resistant than in the insulin-sensitive group (Fig. 2). The % increase in CCL2 gene expression was 60 ± 22% in insulin-sensitive and 169 ± 59% in insulin-resistant subjects ($P = 0.08$). Similarly, the protein concentration of MCP-1 in adipose tissue increased significantly by insulin in the insulin-resistant group but remained unchanged in the insulin-sensitive group (Fig. 3). CCL2 expression and MCP-1 protein concentration in adipose tissue were significantly correlated ($r = 0.53$, $P = 0.008$). In the 8 subjects who received a 6-h saline infusion, expressions of CCL2 and CCL3 remained unchanged (data not shown). Adipocyte size correlated significantly with the mRNA concentration of CD68 ($r = 0.63$, $P = 0.024$).

Whole-body insulin sensitivity was inversely correlated with basal expression of macrophage markers as follows: CD68 (Spearman's $r = -0.58$, $P < 0.01$), EMR1 ($r = -0.62$, $P < 0.01$), ITGAM ($r = -0.50$, $P < 0.05$), ADAM8 ($r = -0.42$, $P = 0.058$, NS). After adjustment for BMI and age, these correlations were as follows: CD68 ($r = -0.44$, $P < 0.05$), EMR1 ($r = -0.28$, NS), ITGAM ($r = -0.67$, $P < 0.001$), ADAM8 ($r = -0.21$, NS). Whole-body insulin sensitivity correlated with CCL2 ($r = -0.60$, $P < 0.01$) and CCL3 ($r = -0.73$, $P < 0.001$) expression in adipose tissue at 6 h.

Serum MCP-1 and MIP-1α concentration. At baseline, serum MCP-1 concentrations were comparable in the insulin-sensitive and insulin-resistant groups (291 ± 19 vs. 343 ± 34 pg/ml, insulin-sensitive vs. insulin-resistant, NS). Insulin decreased serum MCP-1 concentration significantly in the insulin-sensitive group but not in the insulin-resistant group (Fig. 3).

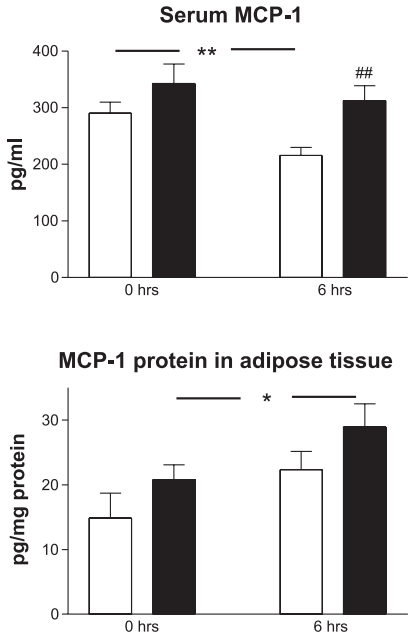

Fig. 3. Serum concentration of MCP-1 (*top*) and MCP-1 protein concentration in adipose tissue in IS (open bars) and IR (filled bars) subjects at 0 and 6 h during euglycemic hyperinsulinemia (rate of continuous insulin infusion 1 mU·kg−1·min−1). **$P < 0.01$ for change between time points, ##$P < 0.01$ for IS vs. IR.

At 6 h, serum MCP-1 concentration was significantly lower in the insulin-sensitive than in the insulin-resistant group (216 ± 14 vs. 313 ± 26 pg/ml, $P < 0.01$ insulin-sensitive vs. insulin-resistant). Serum MIP-1α was under the detection limit of the assay (46.9 pg/ml) in all subjects.

DISCUSSION

The present data are the first to compare responses of the chemokines MCP-1 and MIP-1α to insulin in vivo in adipose tissue between insulin-resistant obese and insulin-sensitive lean women. In vivo euglycemic hyperinsulinemia increased gene and protein expression of MCP-1 (CCL2) more in adipose tissue and decreased MCP-1 less in serum in insulin-resistant than in insulin-sensitive subjects. We also found that whole-body insulin resistance was closely correlated with increased expression of the monocyte/macrophage markers. An increase in basal MCP-1 gene and protein expression has previously been documented in human adipocyte cultures from obese compared with lean subjects (1, 4), and in adipose tissue of ob/ob mice (16). Regarding expression of CCL2 in adipose tissue, we have previously shown that CCL2 correlates significantly with the concentration of MCP-1 protein in adipose tissue (9).

Classically, insulin resistance has been defined as a defective response of an insulin-sensitive gene, protein, or pathway to insulin. In recent years, data from animal studies have suggested that this concept needs to be expanded. Insulin not only regulates molecules and pathways that normally enhance insulin sensitivity, but also molecules that normally confer insulin resistance. Examples of such molecules include TNF-α (8, 23), 11β-HSD-1 (10, 20), and PAI-1 (15) and SREBP-1c (17), which continue to respond to insulin in insulin-resistant states. In the present study, we found CCL2 gene expression in adipose tissue to hyperrespond to insulin in the insulin-resistant subjects (Fig. 2). This finding resembles the transient hyperresponse to insulin (peaking at 1 h in db/db and at 3 h in wild-type mice) described in 3T3-L1 adipocytes made insulin resistant by TNF-α treatment in vitro (16). The insulin-resistant 3T3-L1 adipocytes also overproduced MCP-1 protein. In vivo, insulin increased MCP-1 mRNA concentrations in adipose tissue of ob/ob mice more than in wild-type mice. There was also a transient increase after 1 h of insulin injection in MCP-1 protein in plasma in the ob/ob mice, which was lacking from the wild-type insulin sensitive mice, in which serum MCP-1 levels remained unchanged for 6 h (16). The present data showing an exaggerated increase in CCL2 gene and protein expression in adipose tissue in insulin-resistant obese compared with insulin-sensitive lean subjects are consistent with data in mice. However, in serum, MCP-1 decreased significantly in the control group, consistent with another study also showing a decrease in serum MCP-1 by insulin but not by saline in vivo (5). We did not detect any change in serum MCP-1 in the insulin-resistant group when the level was measured at 0 and 6 h. The data suggest that adipose tissue is not the main determinant of circulating MCP-1 levels in humans. Consistent with this, a catheterization study in humans found no release of MCP-1 from subcutaneous adipose tissue to the circulation (4). Serum MIP-1α levels were undetectable in all subjects. In a study using the same assay, only 15% of the type 2 diabetic patients had detectable concentration of MIP-1α in serum (12).

We also confirm increased basal expression of the macrophage markers CD68 in human adipose tissue in insulin-resistant subjects (1, 22) and report an increase in EMR1. CD68 (3), ADAM-8 (24), and EMR1 (11) are macrophage-specific markers, and ITGAM is a member of an integrin family mediating leukocyte adhesion and migration processes (18). This increase in macrophage gene expression is likely to reflect an increase in macrophage number based on previous data (22), including our own (9). Obesity per se may be a confounding factor when interpreting the data regarding associations to insulin sensitivity. However, in the present study there were also two overweight (BMI 27–30 kg/m2) subjects and one obese subject in the insulin-sensitive group. None of the insulin-resistant subjects had a BMI < 27 kg/m2.

The present study did not address the molecular mechanisms explaining how a gene can hyperrespond to insulin despite whole-body insulin resistance, which reflect mostly insulin resistance at the level of skeletal muscle (13). It is of interest in this respect that insulin inhibition of hormone-sensitive lipase in macrophages, unlike in adipocytes, does not involve activation of the PI 3-kinase pathway (14). In ob/ob mice, macrophages are characterized by reduced insulin receptor expression and signaling, which leads to upregulation of, e.g., the proatherogenic CD36, a scavenger receptor-accumulating lipid. It has recently been demonstrated that macrophages are localized to dead adipocytes (2) and that the rate of adipocyte death is dramatically increased in obesity. Consistent with these data, macrophage marker CD68 expression was significantly correlated with adipocyte size in the present study. Free lipid droplets of dead adipocytes are engulfed by macrophages, which become multinucleated giant cells (2). Against this background, the ability of hyperinsulinemia to promote inflammation in adipose tissue might be viewed as a protective mechanism which help to facilitate removal of dead adipocytes.

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ORIGINAL ARTICLE

Increased expression of the macrophage markers and of 11β-HSD-1 in subcutaneous adipose tissue, but not in cultured monocyte-derived macrophages, is associated with liver fat in human obesity

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Objective: To determine whether increased expression of macrophage markers and of inflammatory markers in subcutaneous adipose tissue is associated with liver fat in human obesity. We also determined whether expression of TNF (gene encoding TNF-α), HSD11B1 (gene encoding 11β-HSD-1) and RETN (gene encoding resistin) in cultured monocyte-derived macrophages differs between obese/overweight and non-obese subjects.
Design: Cross-sectional comparison of obese/overweight and non-obese subjects with respect to adipose tissue gene expression, gene expression in monocyte-derived macrophages, liver fat content and *in vivo* insulin sensitivity.
Subjects: Adipose tissue gene expression, gene expression in monocyte-derived macrophages, liver fat content and *in vivo* insulin sensitivity: 10 healthy non-obese ($24.2 \pm 1.0\,kg/m^2$) and 10 healthy obese/overweight ($33.1 \pm 1.7\,kg/m^2$) women. Gene expression in monocyte-derived macrophages: seven healthy non-obese ($22.1 \pm 0.7\,kg/m^2$) and seven healthy obese/overweight ($36.9 \pm 2.2\,kg/m^2$) women.
Measurements: Adipose tissue biopsies and blood samples for isolation of peripheral mononuclear cells were taken after an overnight fast. Liver fat content was measured using magnetic resonance proton spectroscopy. Whole body insulin sensitivity was measured using the hyperinsulinemic euglycemic clamp technique. Expression levels of TNF, HSD11B1, RETN and the macrophage markers CD68 and ITGAM were determined by real-time PCR.
Results: In adipose tissue, expression of HSD11B1, ITGAM and CD68 was significantly increased in the obese/overweight as compared to the non-obese group. Expression of all these genes was closely positively correlated with liver fat content and inversely correlated with whole body insulin sensitivity. The associations between expression of CD68, ITGAM and HSD11B1 and liver fat were independent of obesity. There were no differences in TNF, HSD11B1, RETN or CD68 gene expression basally or after stimulation with lipopolysaccharide in monocyte-derived macrophages between obese/overweight and non-obese subjects.
Conclusion: Accumulation of fat in the liver is associated with increased adipose tissue inflammation independent of obesity.
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Keywords: adipokines; cytokines; inflammation; insulin resistance; ITGAM; liver fat

Introduction

Several studies in genetically engineered animal models have documented that lack of subcutaneous and visceral fat is associated with severe insulin resistance and fat accumulation in insulin-sensitive tissues.[1] For example, A-ZIP/F-1 mice, which have no white adipose tissue subcutaneously or elsewhere, have dramatically reduced brown fat, severe hepatosteatosis, diabetes, and elevated glucose, insulin, triglyceride and free fatty acid concentrations. Transplantation of wild-type adipose tissue into these mice reverses hyperglycemia, decreases insulin concentrations, corrects insulin signaling defects and normalizes fat content in the liver and muscle.[2] This implies that

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fat accumulation in insulin-sensitive tissues indeed can cause insulin resistance, although the stored triglyceride itself is likely to be inert. These data in mice appear relevant for humans since highly active antiretroviral therapy-associated lipodystrophy as well as more rare forms of human lipoatrophies have uniformly been associated with a fatty liver.[3]

Although fat accumulation in the liver seems important for the development of insulin resistance, other studies suggest that myeloid cells are a prerequisite for development of insulin resistance in insulin-sensitive tissues. Mice lacking IκB kinaseβ, a central coordinator of inflammatory responses through activation of NF-κB in myeloid cells (macrophages and neutrophils), are protected from insulin resistance in skeletal muscle and the liver.[4] In humans, the number of macrophages and expression of macrophage markers such as CD68 are increased in obese and lipoatrophic insulin-resistant subjects.[5–7] Macrophages are the major source of both IL-6 and tumor necrosis factor α (TNF-α) in adipose tissue.[6,8–11] These cytokines can induce insulin resistance locally in adipose cells via downregulation of adipokines such as adiponectin[12,13] which is abundantly secreted systematically and has insulin-sensitising properties especially in the liver.[14,15] Expression of the enzyme converting cortisone to cortisol, 11β-HSD-1, is also increased in insulin-resistant obese subjects.[16,17] This enzyme is expressed in both adipocytes and macrophages.[18,19] Its overexpression in mice results in insulin resistance and visceral obesity.[20] Human fat cells, unlike those of mice, do not produce resistin[21] but resistin has been suggested to be produced by human peripheral blood mononuclear cells.[22] Unlike in mice, resistin may not, however, be related to insulin sensitivity in humans.[23,24]

Until now, only one study has explored the possibility that adipose tissue inflammation is related to hepatic lesions in obesity in humans.[25] This study included a group of morbidly obese nondiabetic and diabetic subjects. The number of macrophages in omental but not subcutaneous fat was found to be correlated with histological liver pathology other than hepatic steatosis. Insulin sensitivity was not directly quantitated. There are no studies examining the relationship between adipose tissue inflammation and liver fat content in non-obese and nonmorbidly obese subjects.

In the present study, we determined whether expression of the macrophage markers and of TNF and HSD11B1 in subcutaneous adipose tissue is related to liver fat content measured by proton spectroscopy and to whole body insulin sensitivity measured by the euglycemic insulin clamp technique. We also determined whether such relationships are independent of obesity. Since it has been suggested that susceptibility genes for insulin action may even reside in myeloid cells,[4] we also compared expression of TNF, HSD11B1 and gene encoding resistin (RETN) in cultured blood monocyte-derived macrophages.

Methods

Subjects and study designs

Subjects. To determine the relationship between adipose tissue inflammation, liver fat content and insulin sensitivity, 20 nondiabetic apparently healthy Caucasian women were recruited based on the following inclusion criteria: (1) age 18–60 years, (2) no known acute or chronic disease other than obesity based on history, physical examination and standard laboratory tests (blood counts, serum creatinine, thyroid-stimulating hormone, electrolyte concentrations and electrocardiogram). Other exclusion criteria included pregnancy or treatment with drugs that may alter glucose tolerance. In each subject, a needle biopsy of adipose tissue was taken for measurement of gene expression and liver fat content was measured with proton spectroscopy and whole body insulin sensitivity using the euglycemic insulin clamp technique (insulin infusion rate 1 mU/kg/min for 2 h). In addition, a separate blood sample was taken for isolation of peripheral blood monocytes. The subjects were divided based on their median body mass index (BMI) into obese/overweight (BMI >28 kg/m^2, $n=10$) and non-obese (BMI <28 kg/m^2, $n=10$) groups. Gene expression of TNF, HSD11B1, CD68 and ITGAM was measured in adipose tissue and in unstimulated monocyte-derived macrophages. These subjects also participated in a study[26] examining effects of a 6 h insulin infusion on adipose tissue gene expression measured at 0, 3 and 6 h. Data on liver fat and monocyte-derived macrophages are novel. In addition to these measurements, gene expression of TNF, HSD11B1 and RETN was measured basally and after 3 and 6 h of lipopolysaccharide (LPS) stimulation in 14 women, which were recruited using the same inclusion and exclusion as mentioned above and divided into obese/overweight (BMI >28 kg/m^2, $n=7$) and non-obese (BMI <28 kg/m^2, $n=7$) groups. An age-matched pair (one obese/overweight and one non-obese subject) was studied on the same day. A blood sample was taken for differentiation of peripheral blood monocytes into macrophages (see below). Macrophages were stimulated with either LPS or saline.

The nature and potential risks of the study were explained to all subjects before obtaining their written informed consent. The ethics committees of the Helsinki University Central Hospital and Karolinska Institutet approved the protocol.

Adipose tissue biopsy and total RNA and cDNA preparation

A needle aspiration biopsy of abdominal subcutaneous fat was taken before insulin infusion under local anesthesia.[27] The fat sample was immediately frozen and stored in liquid nitrogen until analysis. Frozen fat tissue samples (50–150 mg) were homogenized in 2 ml RNA STAT-60 (Tel-Test, Friendswood, TX, USA) and total RNA isolated according to the manufacturer's instructions. After DNase treatment (RNase-free DNase set, Qiagen, Hilden, Germany), RNA

was purified using the RNeasy minikit (Qiagen, Hilden, Germany). RNA concentrations were measured using the RiboGreen fluorescent nucleic acid stain (RNA quantification kit, Molecular Probes, Eugene, OR, USA) and Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). The quality of RNA was checked by agarose gel electrophoresis. Isolated RNA was stored at $-80^{\circ}C$ until qualification of the target mRNAs. A total of 0.1 μg RNA was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, UK) and oligo $(dT)_{12-18}$ primers.

Quantification of mRNA concentrations
mRNA expression levels of TNF, HSD11B1, CD68, RETN and ribosomal protein large P0 (RPLP0) were quantified by real-time PCR using the ABI 7000 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA, USA). cDNA synthesized from 15 ng of total RNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and a gene-specific primer and probe mixture (predeveloped TaqMan Gene Expression Assays, Applied Biosystems). The assays used were TNF, Hs00174128_m1; HSD11B1, Hs00194153_m1; CD68, Hs00154355_m1; ITGAM, Hs00355885_m1; RETN, Hs00220767_m1 and RPLP0, Hs99999902_m1. All samples were run in duplicate. Relative expression levels were determined using a five-point serially diluted standard curve, generated from cDNA from either human adipose tissue or monocyte-derived macrophages (depending on the samples being analyzed). Expression levels were expressed in arbitrary units and normalized relative to the housekeeping gene RPLP0 to compensate for differences in cDNA loading. The levels of RPLP0 were comparable between non-obese and obese subjects in both studies.

Liver fat content
Liver fat content was measured using magnetic resonance proton spectroscopy as previously described.[28]

Whole body insulin sensitivity
Whole body insulin sensitivity was measured using the insulin clamp technique.[29] The study started at 0730 after an overnight fast. Two 18-gauge catheters (Venflon; Viggo-Spectramed, Helsingborg, Sweden) were inserted, one in an antecubital vein for infusion of insulin and glucose, and another retrogradely in a heated hand vein to obtain arterialized venous blood for measurement of glucose concentrations every 5 min and serum-free insulin concentration every 30 min. Regular human insulin (Insulin Actrapid, Novo Nordisk, Denmark) was infused in a primed-continuous fashion. The rate of the continuous insulin infusion was 1 mU/kg/min for 2 h. Normoglycemia was maintained by adjusting the rate of a 20% glucose infusion based on plasma glucose measurements from arterialized venous blood every 5 min. Whole body insulin sensitivity was determined from the glucose infusion rate, corrected for changes in glucose pool size (*M*-value), required to maintain normoglycemia between 30 and 120 min.[29] The *M*-value was expressed as mg/kg fat free mass per per minute.

Isolation and culture of macrophages
Preparation of macrophage monolayers. For the *in vitro* studies assessing basal and LPS-stimulated gene expression, blood was drawn after an overnight fast. Human monocytes were isolated from buffy coats by centrifugation in Ficoll-Paque (Amersham Bioscience AB, Uppsala, Sweden) gradient as described.[30] Washed cells were suspended in Macrophage-SFM Medium (Gibco, Invitrogen Corporation, Grand Island, NY, USA) containing streptomycin and penicillin (Sigma, St Louis, MO, USA). The monocytes were counted, and seeded on plates. After 1 h, nonadherent cells were removed and granulocyte monocyte colony stimulating factor (GM-CSF) (5 ng/ml, Sigma) was added. Monocytes were then cultured for up to 7 days for differentiation into macrophages.

Stimulation of macrophages
In study II, 10 μl of LPS (10 μg/ml, LPS of *Salmonella typhimurium*, Sigma) or saline per 1 ml of SFM Medium/GM-CSF was added to stimulate cultured macrophages for 3 and 6 h at $+37^{\circ}C$ in an atmosphere containing 5% CO_2.

Macrophage mRNA analysis
Macrophage mRNA analysis was performed from cell lysates similarly as described for adipose tissue.

Other measurements
Blood samples were taken after an overnight fast for measurement of plasma glucose, serum insulin, HbA_{1C}, serum alanine aminotransferase concentrations, serum triglyceride, total cholesterol, high-density lipoprotein (HDL) and LDL low-density lipoprotein cholesterol concentrations. The percent of body fat was determined by bioelectrical impedance analysis (BioElectrical Impedance Analyzer System model #BIA-101A; RJL Systems, Detroit, MI, USA).[31] Waist circumference was measured midway between spina iliaca superior and the lower-rib margin, and hip circumference at the level of the greater trochanters.[32]

Analytical procedures
Plasma glucose concentrations were measured in duplicate with the glucose oxidase method using Beckman Glucose analyzer II (Beckman Instruments, Fullerton, CA, USA).[33] Serum-free insulin concentrations were measured using the Auto-DELFIA kit from Wallac (Turku, Finland). HbA_{1C} was

measured by high-pressure liquid chromatography using the fully automated Glycosylated Hemoglobin Analyzer System (BioRad, Richmond, CA, USA).[34] Serum total cholesterol, HDL cholesterol and triglyceride concentrations were measured with enzymatic kits from Roche Diagnostics (Roche Diagnostics Hitachi 917, Hitachi Ltd, Tokyo, Japan) using an autoanalyzer. LDL cholesterol concentration was calculated using the formula of Friedewald *et al*.[35]

Statistical analyses

Obese/overweight and non-obese groups were compared using the Mann–Whitney test. Effects of LPS were analyzed using Friedman's test followed by Dunn's *post hoc* test to compare single measurements. Spearman's rank correlations were calculated. A *P*-value of less than 0.05 was considered statistically significant. Multiple linear regression analysis was performed after logarithmic transformation of data if necessary. The calculations were performed using GraphPad Prism version 3.0 (GraphPad Inc., San Diego, CA, USA) and SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). All data are shown as mean ± standard error of mean (s.e.m.).

Results

Subject characteristics

Characteristics of the 10 non-obese and 10 obese/overweight subjects, whose adipose tissue gene expression, gene expression in monocyte-derived macrophages, liver fat content and *in vivo* insulin sensitivity were measured, are given in Table 1. Liver fat content and markers of insulin resistance, including serum fasting insulin and triglyceride concentrations were higher and the HDL cholesterol concentration was lower in the obese/overweight compared to the non-obese group (Figure 1). Liver fat content averaged 6.8 ± 2.5% in obese/overweight and 0.8 ± 0.1% in non-obese subjects ($P<0.05$). Whole body insulin sensitivity (*M*-value) was 35% lower in the obese/overweight than the non-obese group (5.2 ± 0.7 vs 8.0 ± 0.5 mg/kg fat free mass per minute, obese/overweight vs non-obese, $P<0.01$). Characteristics of the non-obese and obese/overweight subjects whose macrophage gene expression was measured after LPS stimulation are also given in Table 1.

Adipose tissue gene expression

In adipose tissue, the expression of CD68 (1.2 ± 0.1 vs 0.7 ± 0.1, obese/overweight vs non-obese, $P<0.01$), ITGAM (2.3 ± 0.4 vs 1.3 ± 0.2, obese/overweight vs non-obese, $P<0.05$) and HSD11B1 (0.6 ± 0.1 vs 0.3 ± 0.1, obese/overweight vs non-obese, $P<0.05$) was significantly higher (1.7-fold, 1.8-fold and 2.0-fold, respectively) in the obese/overweight than the non-obese group. Adipose tissue TNF expression was slightly, but not significantly, increased in the obese/overweight as compared to the non-obese group (1.4 ± 0.3 vs 1.0 ± 0.2, obese/overweight vs non-obese, NS).

Relationship between markers of adipose tissue inflammation, liver fat and insulin sensitivity

The relationships between adipose tissue gene expression and measures of whole body insulin sensitivity, liver fat content and BMI are shown in Figure 2. Both CD68 and HSD11B1 expression in adipose tissue were inversely correlated with whole body insulin sensitivity and positively correlated with percent liver fat and MBI (Figure 2). ITGAM expression in adipose tissue correlated positively with percent liver fat ($r=0.67$, $P<0.01$). Adipose tissue CD68 expression also correlated positively with those of TNF and HSD11B1 (Figure 3). The expression of two macrophage markers in adipose tissue, CD68 and ITGAM, was closely correlated ($r=0.81$, $P<0.0001$). In multiple linear regression analysis, liver fat was independent of BMI and significantly associated with expression of CD68, ITGAM and HSD11B1 in adipose tissue (Table 2).

Table 1 Clinical and biochemical characteristics of the non-obese and obese/overweight groups

Variable	*Non-obese*[a] (n = *10*)	*Obese/overweight*[a] (n = *10*)	*Non-obese*[b] (n = *7*)	*Obese/overweight*[b] (n = *7*)
Age (years)	34 ± 3	39 ± 4	41 ± 3	41 ± 3
BMI (kg/m^2)	24.2 ± 1.0	33.1 ± 1.7***	22.1 ± 0.7	36.9 ± 2.2***
Waist (cm)	85 ± 3	102 ± 3**	74 ± 2	112 ± 4***
Hip (cm)	97 ± 3	114 ± 3**	95 ± 1	122 ± 4***
HbA_{1c} (%)	5.1 ± 0.1	5.4 ± 0.2	5.3 ± 0.1	5.6 ± 0.3
fS-insulin (mU/l)	4 ± 1	9 ± 1**	3 ± 1	11 ± 2*
fP-glucose (mmol/l)	5.2 ± 0.1	5.5 ± 0.2	4.6 ± 0.2	5.1 ± 0.3
Liver fat (%)	0.8 ± 0.1	6.8 ± 2.5*	ND	ND
S-ALAT (U/l)	23 ± 4	34 ± 6	16 ± 2	27 ± 4*
fS-triglycerides (mmol/l)	0.88 ± 0.10	1.39 ± 0.17*	0.68 ± 0.07	1.53 ± 0.25*
fS-HDL cholesterol (mmol/l)	1.79 ± 0.08	1.38 ± 0.08**	1.76 ± 0.10	1.22 ± 0.13**
fS-LDL cholesterol (mmol/l)	2.39 ± 0.14	2.96 ± 0.24	2.04 ± 0.21	2.60 ± 0.29

Abbreviations: BMI, body mass index; fP, fasting plasma; fS, fasting serum; HDL, high-density lipoprotein; LDL, low-density lipoprotein; S-ALAT, serum alanine aminotransferase. Data are shown as mean ± s.e.m. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ for non-obese vs obese/overweight subjects. [a]These subjects underwent measurements of liver fat content, whole body insulin sensitivity and adipose tissue gene expression. [b]In these subjects, a blood sample was taken to study gene expression basally and after stimulation with LPS in monocyte-derived macrophages.

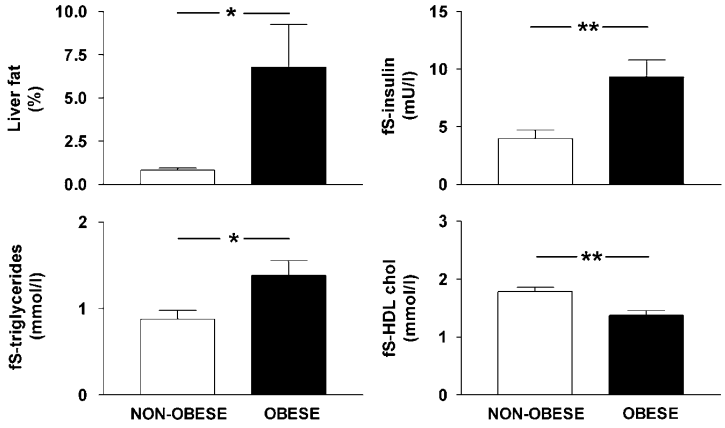

Figure 1 Liver fat content, fasting serum insulin, triglycerides and HDL cholesterol in 10 non-obese (white bars) and 10 obese/overweight (black bars) subjects. *$P<0.05$, **$P<0.001$ for non-obese vs obese/overweight.

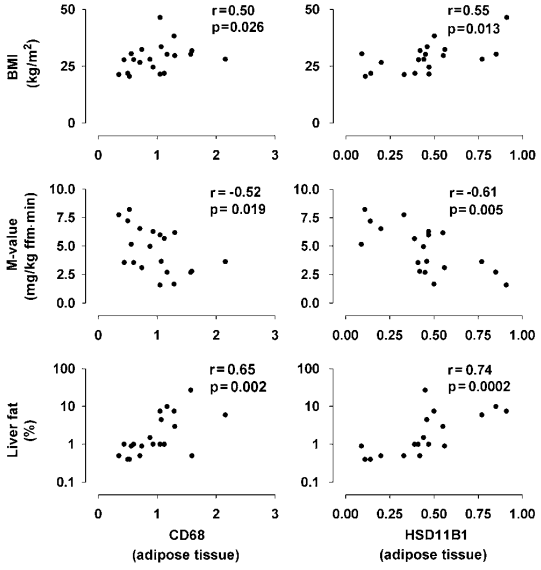

Figure 2 The relationships (*r* denotes Spearman's rank correlation coefficient) between adipose tissue expression of CD68 and HSD11B1 with BMI (upper panels), with whole body insulin sensitivity (*M*-value) (middle panels) and with % liver fat (lower panels). Expression levels are given relative to the housekeeping gene RPLP0.

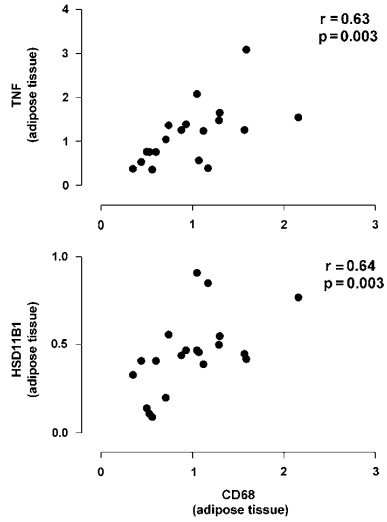

Figure 3 The relationships (*r* denotes Spearman's rank correlation coefficient) between adipose tissue TNF and CD68 expression (upper panel) and between adipose tissue HSD11B1 and CD68 expression (lower panel). Expression levels are given relative to the housekeeping gene RPLP0.

Gene expression in monocyte-derived macrophages

In monocyte-derived macrophages, there were no differences between the obese/overweight and non-obese groups regarding TNF (37.4 ± 12.6 vs 37.8 ± 5.6, obese/overweight vs non-obese, NS), CD68 (1.2 ± 0.1 vs 1.2 ± 0.1, obese/overweight vs non-obese, NS) or HSD11B1 (0.7 ± 0.1 vs 0.5 ± 0.1, obese/overweight vs non-obese, NS) expression. In the obese/overweight and non-obese subjects, whose macrophages were stimulated with LPS, TNF expression increased significantly from basal to 3 h and from basal to 6 h in both non-obese and obese/overweight groups (Figure 4). The increase was similar in both groups. RETN expression remained unchanged with no differences between the groups (Figure 4). LPS also increased HSD11B1 expression

Table 2 Multiple linear regression analysis of associates of liver fat

Dependent variable	*Independent variables*	*Regression coefficient ± s.e.*	P-*value (two-tail)*	*Squared multiple R × 100 (%)*
Liver fat (log)	BMI	0.038 ± 0.01	0.016	58.3
	CD68	0.58 ± 0.19	0.008	
Liver fat (log)	BMI	0.033 ± 0.01	0.025	63.8
	ITGAM (log)	1.10 ± 0.31	0.002	
Liver fat (log)	BMI	0.026 ± 0.02	0.16	53.6
	HSD11B	1.25 ± 0.51	0.022	

Abbreviation: BMI, body mass index.

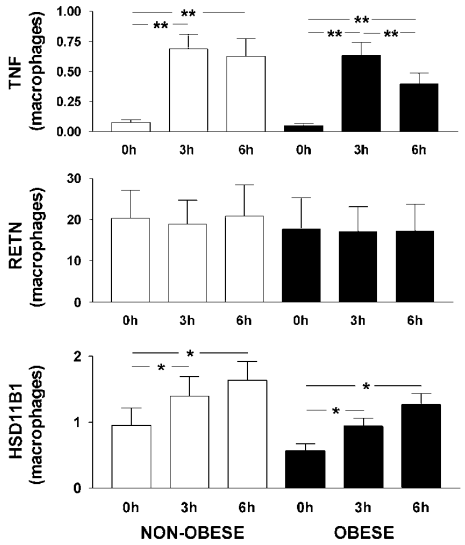

Figure 4 The expressions of TNF, RETN and HSD11B1 in monocyte-derived macrophages at 0, 3 and 6 h after LPS stimulation in non-obese (white bars) and obese/overweight subjects (black bars). $*P<0.05$, $**P<0.01$ for comparison between time points. Expression levels are given relative to housekeeping gene RPLP0.

significantly and similarly in the obese/overweight and non-obese groups (Figure 4). There were no changes in gene expression after saline stimulation.

Discussion

In the present study, we confirmed increased expression of the macrophage markers CD68 and ITGAM, and of HSD11B1 in human adipose tissue in obese/overweight as compared to non-obese subjects. The novel finding was that these changes were associated with liver fat content and whole body insulin sensitivity. These relationships were shown to be independent of obesity. We found no alterations in expression of TNF, HSD11B1 or RETN in monocyte-derived macrophages between obese/overweight and non-obese subjects.

In keeping with most,[16,17,36–41] but not all[42] previous studies, we found expression of HSD11B1 to be significantly increased in obese/overweight subcutaneous adipose tissue. Adipose tissue HSD11B1 mRNA levels correlate closely with 11β-HSD-1 enzyme activity[37,43] and with 11β-HSD-1 protein expression in adipose tissue.[36] 11β-HSD-1 acts predominantly as a reductase *in vivo*, converting inactive cortisone (11-dehydrocorticosterone in mice) into biologically active cortisol (corticosterone in mice).[44] Hence increased HSD11B1 gene expression may increase local corticosteroid concentrations in subcutaneous adipose tissue. Overexpression of HSD11B1 selectively in adipose tissue in transgenic mice results in visceral obesity, insulin resistance, diabetes and hyperlipidemia.[45] The cell type responsible for HSD11B1 overexpression in obesity cannot be determined from the present data.

HSD11B1 knockout mice have lower RETN and TNF mRNA levels in adipose tissue than wild-type mice.[46] If mouse data were applicable to humans, one might have expected TNF expression to be increased in adipose tissue from obese/overweight subjects in the current study. Also, the increased CD68 expression would be expected to be accompanied by a significant increase in TNF expression. Although TNF was increased, the increase was not statistically significant. This could have been a type 2 error or, alternatively, due to phenotypic differences in macrophages. As reviewed elsewhere,[47,48] activated macrophages produce high amounts of proinflammatory cytokines such as TNF-α while apoptotic cell engulfment appears to signal macrophages to transform into a reparative population which expresses less proinflammatory cytokines.[49] Regarding resistin, we confirm resistin expression in human macrophages[50] but found no difference in resistin expression in macrophages between obese/overweight and non-obese subjects. Although resistin has proinflammatory properties and stimulates TNF-α and IL-6 production in human peripheral blood mononuclear cells,[22] RETN expression in adipose tissue or muscle does not differ between insulin-resistant and insulin-sensitive subjects.[51–53]

Expression of the macrophage marker CD68 and the percentage of CD68-positive cells has been shown to be increased in obese adipose tissue,[5,6] but CD68 expression has previously neither been shown to be related to whole body insulin resistance or liver fat content, nor to expression of TNF and HSD11B1 in adipose tissue. These relationships may reflect a role of adipose tissue inflammation, rather than

peripheral fat, in regulating hepatic insulin sensitivity, since liver fat and the number of adipose tissue macrophages are also increased in patients with subcutaneous lipoatrophy.[28,54,55] In obese mice, deletion of either the monocytes chemoattractant protein-1 (MCP-1 or CCL2) gene [56] or its receptor (CCR2)[57] are associated with reduction in the number of macrophages in adipose tissue and liver fat content. Only one previous human study has examined the relationship between adipose tissue inflammation and liver abnormalities. Cancello *et al*.[25] found no relationship between the number of macrophages in subcutaneous adipose tissue and liver fat content in morbidly obese subjects. The number of macrophages in omental adipose tissue correlated with liver histologic lesions but not with liver fat.[25] No healthy lean controls were included in this study. A limitation of our study is that we measured gene expression of two macrophage markers but could not perform immunohistochemical studies because a needle rather than surgical biopsy of adipose tissue was taken.

The studies showing macrophage infiltration in human and murine adipose tissue[5,6] and that mice lacking IκB kinaseβ are protected from insulin resistance in skeletal muscles and the liver[4] raised the interest in exploring the role of myeloid cells in the pathogenesis of insulin resistance in humans. Early studies suggested that insulin receptor tyrosine kinase activity is low in freshly isolated monocytes from non-obese insulin-resistant as compared to sensitive subjects.[58] Mononuclear cell adhesion to the endothelium has also been found to correlate with the degree of insulin resistance.[59] Recently, freshly isolated circulating monocytes were suggested to be in a proinflammatory state in obese subjects compared to normal-weight controls.[60] These data do not necessarily disagree with the present findings of unaltered basal and stimulated expression of insulin resistance genes in monocyte-derived macrophages, since we did not study freshly isolated monocytes in which gene expression reflects both acquired and genetic factors. Obviously this warrants isolation of macrophages from adipose tissue and measurement of a larger number of genes as well as a direct assessment of the macrophage fraction.

In conclusion, in keeping with previous data the expression of macrophage markers is increased in obese human adipose tissue. This sign of inflammation is correlated with liver fat content independent of obesity. Liver fat content in turn is correlated with serum insulin and other signs of insulin resistance. These data may help to understand why only some but not all obese subjects develop signs of insulin resistance.

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IV

Original Article

Adipose Tissue Inflammation and Increased Ceramide Content Characterize Subjects With High Liver Fat Content Independent of Obesity

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OBJECTIVE—We sought to determine whether adipose tissue is inflamed in individuals with increased liver fat (LFAT) independently of obesity.

RESEARCH DESIGN AND METHODS—A total of 20 nondiabetic, healthy, obese women were divided into normal and high LFAT groups based on their median LFAT level (2.3 ± 0.3 vs. 14.4 ± 2.9%). Surgical subcutaneous adipose tissue biopsies were studied using quantitative PCR, immunohistochemistry, and a lipidomics approach to search for putative mediators of insulin resistance and inflammation. The groups were matched for age and BMI. The high LFAT group had increased insulin (P = 0.0025) and lower HDL cholesterol (P = 0.02) concentrations.

RESULTS—Expression levels of the macrophage marker CD68, the chemokines monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α, and plasminogen activator inhibitor-1 were significantly increased, and those of peroxisome proliferator–activated receptor-γ and adiponectin decreased in the high LFAT group. CD68 expression correlated with the number of macrophages and crown-like structures (multiple macrophages fused around dead adipocytes). Concentrations of 154 lipid species in adipose tissue revealed several differences between the groups, with the most striking being increased concentrations of triacylglycerols, particularly long chain, and ceramides, specifically Cer(d18:1/24:1) (P = 0.01), in the high LFAT group. Expression of sphingomyelinases SMPD1 and SMPD3 were also significantly increased in the high compared with normal LFAT group.

CONCLUSIONS—Adipose tissue is infiltrated with macrophages, and its content of long-chain triacylglycerols and ceramides is increased in subjects with increased LFAT compared with equally obese subjects with normal LFAT content. Ceramides or their metabolites could contribute to adverse effects of long-chain fatty acids on insulin resistance and inflammation. ***Diabetes*** **56:1960–1968, 2007**

Recent studies have shown that the fatty liver is insulin resistant and overproduces many, if not most, of the cardiovascular risk factors associated with the metabolic syndrome (1). Insulin resistance in the liver results in VLDL overproduction, which leads to hypertriglyceridemia and low HDL cholesterol levels (2). The liver, once fatty, also overproduces fibrinogen, von Willebrand factor, C-reactive protein (3), and plasminogen activator inhibitor (PAI)-1 (3,4). Resistance to insulin inhibition of hepatic glucose production basally and postprandially results in hyperglycemia and hyperinsulinemia (1). Abdominal obesity is more common in individuals with a fatty liver than in those without, and intra-abdominal fat correlates with liver fat (LFAT) (5,6). However, the amount of fat in the liver is poorly correlated with subcutaneous obesity (1,5) and predicts the metabolic syndrome (7), type 2 diabetes (8,9), and cardiovascular disease (10) independent of obesity.

Factors regulating LFAT content independent of obesity are poorly understood. Previous studies have shown that adipose tissue in obese subjects is inflamed compared with nonobese subjects (11,12). This inflammation is characterized by an increase in macrophage numbers and expression of macrophage markers such as CD68 (11,12). Expression of insulin-resistant genes and the local production of their protein products by macrophages in adipose tissue are also increased in obese compared with nonobese subjects (13–16). These and other changes may contribute to increased lipolysis and adiponectin deficiency, which characterize adipose tissue of insulin-resistant subjects and may increase LFAT content (17). The stimulus for macrophage accumulation in adipose tissue, on the other hand, is unclear. The first studies comparing obese and nonobese subjects showed macrophage accumulation to be directly related to cell size (11). Recently, Cinti et al. (18) showed that in obese compared with nonobese subjects, macrophages surround dead adipocytes, and they suggested that adipocyte cell death could serve as one stimulus for macrophage infiltration.

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LFAT, liver fat; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; PAI, plasminogen activator inhibitor; PPAR, proliferator–activated receptor; TNF-α, tumor necrosis factor-α.

Regarding the causes of adipose tissue inflammation, we have previously shown in two independent studies that the percentage of total fat in diet is positively correlated with LFAT content (19,20). This could alter fatty acid composition of adipose tissue (21). Recent advances in ultra-performance liquid chromatography combined with mass spectrometry methodology and data processing have enabled structural characterization and quantitation of hundreds of lipid species in minute biological samples (22,23). Such methodology has hitherto not been used to study human adipose tissue.

In the present study, we reasoned that fat accumulation in the liver distinguishes between those obese subjects who develop insulin resistance and those who do not. We hypothesized if adipose tissue inflammation is important for fat accumulation in the liver, then adipose tissue should be inflamed in obese subjects who have excess fat in the liver compared with equally obese subjects with normal LFAT content. To examine this, we recruited a group of obese women with a narrow range of BMIs and measured their LFAT content using proton magnetic resonance spectroscopy and body composition using magnetic resonance imaging (MRI) and other techniques. The women were divided into groups with high and normal LFAT content, based on their median LFAT content. The groups were matched with respect to age and body weight. A surgical biopsy of subcutaneous adipose tissue was taken for immunohistochemistry and gene expression studies. Lipidomic analyses were performed using the ultra-performance liquid chromatography combined with mass spectrometry platform.

RESEARCH DESIGN AND METHODS

A total of 20 obese (BMI 30–40 kg/m^2) but otherwise healthy women not using any medications were recruited on the basis of the following inclusion criteria: *1*) aged 18–60 years; *2*) no known major organ system disease determined by history, physical examination, and standard laboratory tests (hemoglobin, sedimentation rate, and electrolytes plasma glucose); *3*) alcohol consumption <20 g/day assessed by a questionnaire (24); *4*) no history of liver diseases; and *5*) stable weight for the last 6 months. All patients had been obese for several years, were physically inactive, and reported consuming a normal Finnish diet.

Patients were studied after an overnight fast. A blood sample was taken for measurement of plasma glucose, serum insulin and C-peptide, serum triglyceride, and total and HDL cholesterol concentrations. A surgical subcutaneous adipose tissue biopsy, yielding ~5 g tissue, was performed under local anesthesia. Part of the biopsy, which was used for gene expression analyses, was immediately frozen in liquid nitrogen and kept at −80°C. Another part of the biopsy was fixed in formalin and embedded in paraffin. Fat cell size was measured by collagenase digestion, as previously described (25). Intrahepatic fat content (percentage LFAT) was determined by magnetic resonance proton spectroscopy, and subcutaneous and abdominal fat areas were measured by MRI. Whole body composition was measured by bioelectrical impedance plethysmography (BioElectrical Impedance Analyzer System, model no. BIA-101A; RJL Systems, Detroit, MI). Waist circumference was measured midway between the spina iliaca superior and the lower rib margin, and hip circumference was measured at the level of the greater trochanters. The nature and potential risks of the study were explained to all subjects before obtaining their written informed consent. The study was approved by the ethics committees of the Helsinki University Central Hospital and the Karolinska Institutet.

Total RNA and cDNA preparation. Frozen adipose tissue (50–150 mg) was homogenized in 2 ml RNA STAT-60 (Tel-Test, Friendswood, TX), and total RNA was isolated according to the manufacturer's instructions, as described. After DNase treatment, RNA was purified using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentrations were measured using the RiboGreen fluorescent nucleic acid stain (RNA quantification kit; Molecular Probes, Eugene, OR). The quality of RNA was analyzed by Agilent Bioanalyzer 2100 (Agilent Technologies). Average yields of total RNA were 3 ± 1 μg per 100 mg adipose tissue wet wt and did not differ between the groups. Isolated RNA was stored at −80°C until the quantification of the target mRNAs. A total of 0.1 μg RNA was transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, U.K.) and oligo $(dT)_{12-18}$ primer.

Quantification of gene expression. mRNA expression of specific genes was quantified by real-time PCR using the ABI 7000 Sequence Detection System instrument and software (Applied Biosystems). cDNA synthesized from 15 ng total RNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and a gene-specific primer and probe mixture (predeveloped TaqMan Gene Expression Assays; Applied Biosystems) in a final volume of 25 μl. The assays used included: Hs00154355_m1 for CD68, Hs00234140_m1 for CCL2, Hs00234142_m1 for CCL3, Hs00605917_m1 for adiponectin, Hs00234592_m1 for peroxisome proliferator–activated receptor (PPAR)-γ, Hs00194153_m1 for 11βHSD1, Hs00174131_m1 for interleukin 6, Hs00174128_m1 for tumor necrosis factor (TNF)-α, Hs00167155_m1 for PAI-1, Hs00609415_m1 for SMPD1, Hs00906924_g1 for SMPD2, Hs00218713_m1 for SMPD3, Hs99999910_m1 for TBP (TATA-box binding protein), and Hs99999902_m1 for RPLP0 (ribosomal protein large P0). All samples were run in duplicate. Relative expression levels were determined using a five-point serially diluted standard curve, generated from cDNA of human adipose tissue. Gene expression was expressed in arbitrary units and normalized relative to the housekeeping genes RPLP0 and TBP to compensate for differences in cDNA loading. The average of these two values was used for normalization.

Immunohistochemistry. Subcutaneous biopsies from all subjects were used for immunohistochemical staining for the study of macrophages and necrotic adipocytes. CD68 was used as a marker for macrophages, and perilipin was used as a negative marker for necrotic adipocytes, as previously described by Cinti et al. (18). Staining was performed using a standard protocol on sections from formalin-fixed paraffin-embedded tissue blocks. Serial sections were microwave-treated in 10 mmol/l citrate buffer (pH 6.0) and then incubated for 1 h at room temperature with primary antibodies, mouse monoclonal anti-CD68 (Novocastra Laboratories, Newcastle upon Tyne, U.K.), polyclonal guinea pig anti-perilipin (Acris, Hiddenhausen, Germany), or with a mouse monoclonal isotypic control (Abcam, Cambridge, U.K.) for CD68. After rinsing in PBS buffer containing 0.25% Triton X-100 (pH 7.2), sections were incubated with secondary biotinylated goat anti-mouse (Dako cytomation; Dako, Glostrup, Denmark) or biotinylated goat anti–guinea pig (Abcam) antibodies. Avidin-biotin peroxidase complexes (Vector Laboratories, Burlingame, CA) were added followed by visualization with 3.3′-diaminobenzidine tetrachloride (Vector). All sections were counterstained with Harris hematoxylin (Histolab, Göteborg, Sweden). For each subject, the numbers of macrophages (identified as CD68+ cells) and crown-like structures within the entire section were counted by two independent observers using a light microscope and normalized for the total section area. A crown-like structure was defined as a perilipin-free adipocyte surrounded by at least three macrophages (18). Measurement of total section area and average adipocyte area (in arbitrary units) was performed using the GNU Image Manipulation Program 2.2 (GIMP 2.2).

Measurement of monocyte chemoattractant protein (MCP)-1 expression in adipose tissue. A frozen sample of human subcutaneous adipose tissue (100–430 mg) was homogenized in lysis buffer (26). The homogenate was centrifuged for 30 min (+4°C, 14,000 rpm) and the supernatant stored at −80°C until measurement of the MCP-1 concentration using the Human CCL2/MCP-1 Immunoassay kit (Quantikine; R&D Systems, Minneapolis, MN) and the Bio Assay Reader HTS 7000 Plus (Perkin Elmer, Norwalk, CT). Total protein was measured using the BC Assay-protein quantitation kit (Uptima Interchim, Montlucan, France).

Lipidomics/adipose tissue sample preparation. Approximately 20 mg adipose tissue was weighed, and 20 μl of an internal standard mixture and 40 μl NaCl (0.9%) were added to the sample. Lipids were extracted from the samples with 200 μl chloroform:methanol (2:1) solvent, and the tissue was homogenized with a glass rod. After vortexing for 2 min and incubating for 1 h at room temperature, the lower layer (~100 μl) was separated by centrifugation at 10,000 rpm for 3 min at room temperature. Labeled standard mixture (20 μl) was added to the lipid extract. Internal and external standards are listed in the supplemental materials (found in the online appendix [available at http://dx.doi.org/10.2337/db07-0111]).

Lipidomic analysis. Lipid extracts were analyzed on a Q-ToF Premier mass spectrometer (Waters, Milford, MA) combined with an Acquity ultra-performance liquid chromatography. The column was an Acquity ultra-performance liquid chromatography bridged ethyl hybrid C18 10 × 50 mm with 1.7-μm particles and was kept at 50°C. The binary solvent system A included water (1% 1M NH_4Ac, 0.1% HCOOH), and solvent system B included LC/MS grade (Rathburn) acetonitrile/isopropanal (5:2, 1% 1M NH_4Ac, 0.1% HCOOH). The gradient started from 65% A/35% B, reached 100% B in 6 min, and remained there for the next 7 min. The total run time, including a 5-min reequilibration step, was 18 min. The flow rate was 0.2 ml/min, and injection volume was 1 μl. The temperature of the sample

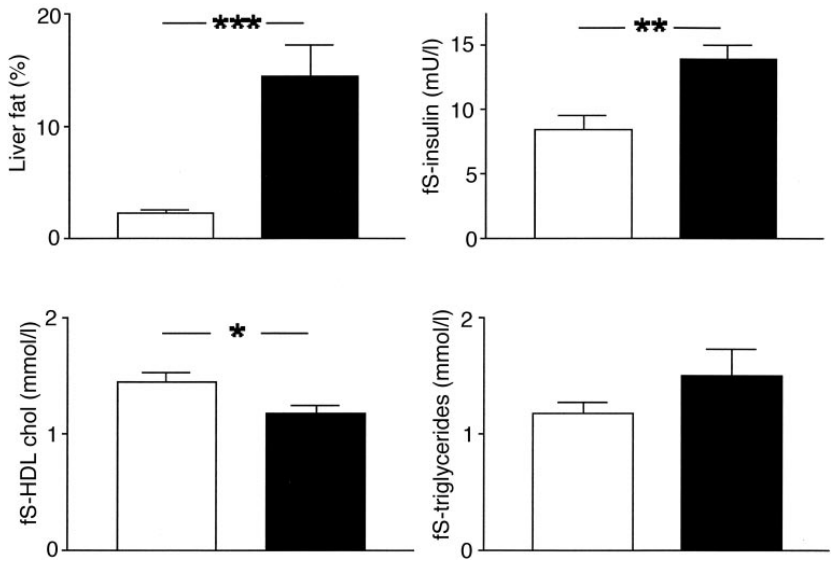

FIG. 1. LFAT percentage and fasting serum (fS) insulin, fS-HDL cholesterol, and fS-triglyceride concentrations in women with normal (□) and high (■) LFAT. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

organizer was set at 10°C. Lipid profiling was carried out using positive ion mode. The data were collected at mass range of charge/mass ratio 300–2,000, with a scan duration of 0.2 s. The source temperature was set at 120°C, and nitrogen was used as desolvation gas (800 l/h) at 250°C. The voltages of the sampling cone and capillary were 39 V and 3.2 kV, respectively. Reserpine (50 μg/l) was used as the lock spray reference compound (5 μl/min), with a 10-s scan frequency.

The obtained data were converted into netCDF file format using Dbridge software from MassLynx (Waters). The converted data were processed using MZmine software version 0.60 (22). Lipids were identified based on their retention time and mass/charge ratio using our in-house built lipid database as previously described (27). All the identified lipids were quantified by normalizing with corresponding internal standards. Sphingomyelins were normalized with GPCho(17:0/17:0) internal standard.

Analytical procedures. Plasma glucose concentrations were measured in duplicate with the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). Serum-free insulin concentrations were measured using the Auto-DELFIA kit from Wallac (Turku, Finland) and C-peptide concentrations by radioimmunoassay. Serum total cholesterol, HDL cholesterol, and triglyceride concentrations were measured with enzymatic kits from Roche Diagnostics, and serum free fatty acids were measured with an enzymatic kit from Wako using an autoanalyzer (Roche Diagnostics Hitachi 917; Hitachi, Tokyo, Japan). The concentration of LDL cholesterol was calculated using the Friedewald formula.

Statistical analysis. Statview (SAS Institute, Cary, NC) software was used to perform the statistical analysis. Physical and biochemical characteristics of the study subjects were analyzed using nonparametrical methods. Groups were compared using the Mann-Whitney test. All correlations were performed using Spearman's rank correlation. Differences in gene expression and lipidomics data were compared by unpaired *t* test after logarithmical transformation of variables (gene expression data only) with non–normal distribution. Statistical significance was assigned to a value of $P < 0.05$. Data are presented as mean ± SEM.

TABLE 1
Physical and biochemical characteristics of the study subjects divided into normal and high LFAT groups

	Normal LFAT	High LFAT	Significance*
n	10	10	—
Age (years)	44 ± 3	37 ± 2	NS
Body weight (kg)	98 ± 2	98 ± 3	NS
BMI (kg/m^2)	35.4 ± 1.1	36.7 ± 0.8	NS
Waist-to-hip ratio	0.98 ± 0.06	0.97 ± 0.03	NS
Whole body fat (%)	39.7 ± 1.0	37.1 ± 1.2	NS (0.08)
Intra-abdominal fat (cm^3)	1,631 ± 167	1,979 ± 261	NS
Subcutaneous fat (cm^3)	7,407 ± 333	6,644 ± 526	NS
Fat mass (kg)	38.7 ± 1.3	36.7 ± 1.9	NS
LFAT (%)	2.3 ± 0.3	14.4 ± 2.9	0.0002
Fasting plasma glucose (mmol/l)	5.1 ± 0.2	5.5 ± 0.2	NS
A1C (%)	5.4 ± 0.1	5.7 ± 0.2	NS
Fasting serum insulin (mU/l)	8.4 ± 1.0	13.9 ± 1.2	0.0025
Fasting serum C-peptide (nmol/l)	0.88 ± 0.07	1.12 ± 0.10	NS (0.08)
Fasting serum LDL cholesterol (mmol/l)	2.6 ± 0.2	3.0 ± 0.2	NS
Fasing serum HDL cholesterol (mmol/l)	1.5 ± 0.1	1.2 ± 0.1	0.02
Fasting serum triglycerides (mmol/l)	1.2 ± 0.1	1.5 ± 0.2	NS
Fasting serum free fatty acids (μmol/l)	669 ± 101	606 ± 76	NS

Data are means ± SEM. *Exact *P* values are shown in parentheses for significances in the 0.05–0.10 range.

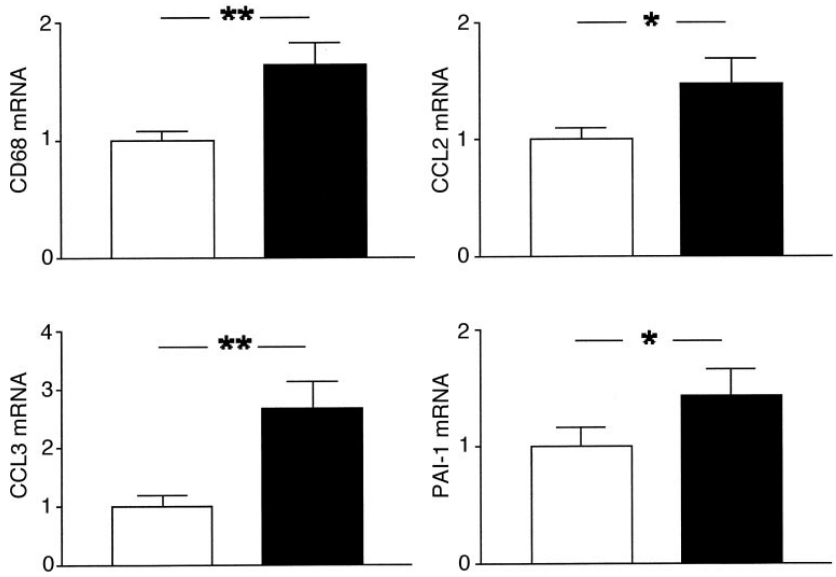

FIG. 2. mRNA expression of CD68, CCL2, CCL3, and PAI-1 in subcutaneous adipose tissue in women with normal (□) and high (■) LFAT. Expression is in arbitrary units normalized for housekeeping genes RPLP0 and TBP. *$P < 0.05$, **$P < 0.01$.

RESULTS

The 20 women were divided into two groups according to their LFAT content: normal LFAT ($n = 10$, range 1–3.5%, mean 2.3 ± 0.3%) and high LFAT ($n = 10$, range 6–34%, mean 14.4 ± 2.9%). Characteristics of the normal and high LFAT groups are listed in Table 1. The groups were similar, with respect to age, sex, BMI, and subcutaneous and intra-abdominal fat masses. Fasting serum insulin concentrations were significantly higher and HDL cholesterol concentrations lower in the high LFAT group compared with the normal LFAT group (Table 1 and Fig. 1).

Expression in subcutaneous adipose tissue of the macrophage markers CD68, CCL2 (MCP-1) and CCL3 (macrophage inflammatory protein [MIP]-1α), and PAI-1 was significantly higher in the high LFAT group compared with the normal LFAT group (Table 2 and Fig. 2). MCP-1 protein expression in adipose tissue correlated with gene expression ($r = 0.53$, $P = 0.023$). Expression of adiponectin and PPAR-γ was significantly lower in the high LFAT group than in the normal LFAT group (Table 2). Expression of 11βHSD1, interleukin 6, and TNF-α did not differ significantly between the groups, although TNF-α tended to be increased in the high LFAT group (Table 2). Expression of the housekeeping genes RPLP0 and TBP did not differ between the groups (RPLP0: 1.9 ± 0.3 vs. 2.1 ± 0.2, NS, and TBP: 4.0 ± 0.5 vs. 4.0 ± 0.4, NS, for normal vs. high LFAT groups, respectively). Expression of the macrophage marker CD68 correlated significantly with LFAT ($r = 0.67$, $P = 0.0035$) but not with BMI ($r = 0.10$, NS) (Fig. 3*B* and *C*).

TABLE 2
Relative gene expression levels in subcutaneous adipose tissue in normal and high LFAT groups

Gene	Normal LFAT	High LFAT	*P*
CD68	1.9 ± 0.1	3.2 ± 0.4	0.003
CCL2	0.35 ± 0.03	0.52 ± 0.08	0.05
CCL3	0.36 ± 0.07	1.0 ± 0.2	0.002
Adiponectin	1.0 ± 0.1	0.9 ± 0.1	0.04
11βHSD1	5.7 ± 1.0	5.6 ± 0.9	NS
IL6	0.12 ± 0.03	0.12 ± 0.02	NS
TNF-α	1.5 ± 0.3	2.4 ± 0.8	NS (0.12)
PAI-1	2.8 ± 0.5	4.0 ± 0.6	0.03
PPAR-γ	1.1 ± 0.09	0.9 ± 0.05	0.04
SMPD1	1.5 ± 0.1	1.9 ± 0.1	0.01
SMPD2	1.1 ± 0.1	1.3 ± 0.1	NS (0.08)
SMPD3	1.3 ± 0.1	1.8 ± 0.2	0.05

Data are means ± SEM. Expression of each gene is normalized to the expression of housekeeping genes TBP and RPLP0.

Adipocyte cell size. The average adipocyte cross-sectional area in the sectioned tissue and fat cell size measured by collagenase digestion did not differ between the normal and high LFAT groups (2,067 ± 109 vs. 2,118 ± 207 arbitrary units, NS, for cross-sectional area and 25.2 ± 0.8 vs. 24.9 ± 1.0 μm for fat cell size, NS).

Immunohistochemistry. Positive staining for the macrophage marker CD68 was seen in 19 of 20 adipose tissue biopsies. The total number of macrophages per section area did not differ between the normal and high LFAT groups (22 ± 6 vs. 37 ± 10, NS, for normal vs. high LFAT groups, respectively) but did correlate significantly with the mRNA expression of CD68 in adipose tissue in all subjects ($r = 0.53$, $P = 0.02$) (Fig. 3*A*). Some macrophages were arranged in crown-like structures (Fig. 4) around necrotic perilipin-free adipocytes (Fig. 5). The number of crown-like structures per section area correlated with borderline significance with the mRNA expression of CD68 ($r = 0.44$, $P = 0.057$).

Lipidomics of adipose tissue. Lipids were analyzed from subcutaneous adipose tissue of normal and high LFAT subjects. A total of 154 lipid molecular species were identified. The differences were dominated by increased ceramides, sphingomyelins, ether phospholipids, and triacylglycerols. No specific trend was observed for ester-linked phospholipids or diacylglycerols. Specifically, all

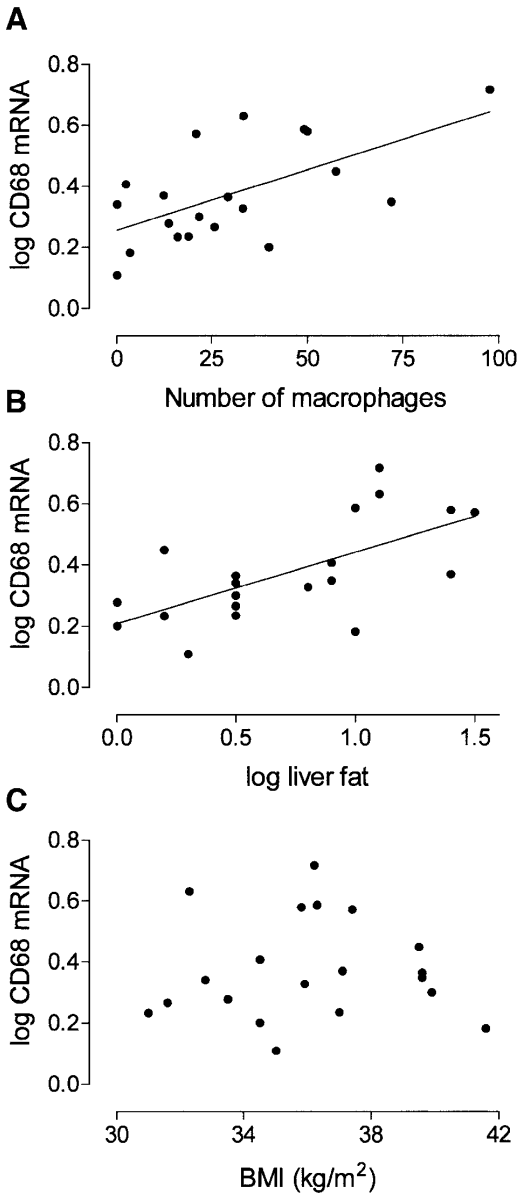

FIG. 3. The relationship between CD68 expression in adipose tissue and the number of macrophages (*A*) ($r = 0.53$, $P = 0.02$), LFAT (*B*) ($r = 0.67$, $P = 0.0035$), and BMI (*C*) ($r = 0.10$, NS).

three identified ceramide molecular species were increased in the high LFAT group, with the most abundant species Cer(d18:1/24:1) being increased 1.5-fold ($P < 0.01$) (Fig. 6*A*). The sphingomyelin molecular species were proportionally increased, although the levels of the most abundant sphingomyelin molecular species SM(d18:1/16:0) did not differ between the normal and high LFAT groups. Expression levels in adipose tissue of the sphingomyelinases SMPD1 and SMPD3 were significantly greater in the high LFAT group compared with the normal LFAT group (Table 2). Most of the 95 identified triacylglycerol species were increased in the high LFAT group. The degree of difference was positively correlated with the triacylglycerol carbon chain length (Fig. 6*B*). No such trend was observed for the degree of fatty acid saturation. Several of the most elevated triacylglycerol species contained an odd number of total fatty acid chain carbons (Fig. 6*B*).

DISCUSSION

This is the first study that has sought to determine whether increased LFAT, independent of obesity, is associated with adipose tissue inflammation as determined by quantitative PCR, immunohistochemistry, and lipidomics approaches. It is also the first study to use a lipidomics approach to analyze adipose tissue in human subjects. We found that several of the changes previously attributed to obesity characterize equally obese subjects who differ with respect to LFAT content. High LFAT was associated with increased expression of CD68, CCL2 (MCP-1), CCL3 (MIP-1α), and PAI-1 and decreased expression of PPAR-γ in adipose tissue. Average fat cell sizes were similar between the groups when measured using both collagenase digestion and morphometry. CD68 expression in adipose tissue correlated with the number of macrophages and crown-like structures when adipose tissue was analyzed by immunohistochemistry. We also found, as has previously been found in obese subjects compared with lean subjects by Cinti et al. (18), that the crown-like structures surrounded perilipin-negative adipocytes. The lipidomics analysis revealed in the high LFAT group an increase in the sphingolipid ceramide and in long-chain triacylglycerols. Although this human study cannot prove cause and effect, ceramide synthesis is stimulated by long-chain fatty acids, and ceramides can induce both inflammation and insulin resistance (28).

We divided the study subjects according to their median LFAT into two groups. These groups were matched with respect to age, sex, BMI, waist circumference, and amounts of intra-abdominal and subcutaneous fat measured with MRI. The highest LFAT content in the normal LFAT group was 3.5% and the lowest content in the high LFAT group 6%. In the Dallas Heart Study (29), in which LFAT was measured in 2,349 subjects using proton magnetic resonance spectroscopy, the upper limit of normal for LFAT was defined as 5.56%. This upper limit for LFAT, when measured with spectroscopy, is similar to that which we have found to correspond to the upper limit of S-alanine aminotransferase in women and men (5). Based on these data, the normal LFAT group in the present study indeed had normal LFAT content, and the high LFAT group had increased LFAT. In keeping with previous data in weight-matched groups (1,30), the subjects with increased LFAT had signs of insulin resistance (higher insulin and lower HDL cholesterol concentrations) compared with those with normal LFAT (Table 1).

Expression of CD68, CCL2 (MCP-1) and CCL3 (MIP-1α), and PAI-1 were significantly increased and PPAR-γ significantly decreased in the high compared with the normal LFAT group (Table 2). Previous studies have documented increases in gene and protein expression of each of these genes in obese subjects compared with nonobese subjects (31–35). The increased expression of MCP-1 is of particular interest because overexpression of CCL2 (MCP-1) in adipose tissue in mice is sufficient to induce insulin resistance and macrophage infiltration and increase triglyceride content (36). CCR2 (CCL2 receptor) deficiency has opposite effects (37). We did not attempt to determine

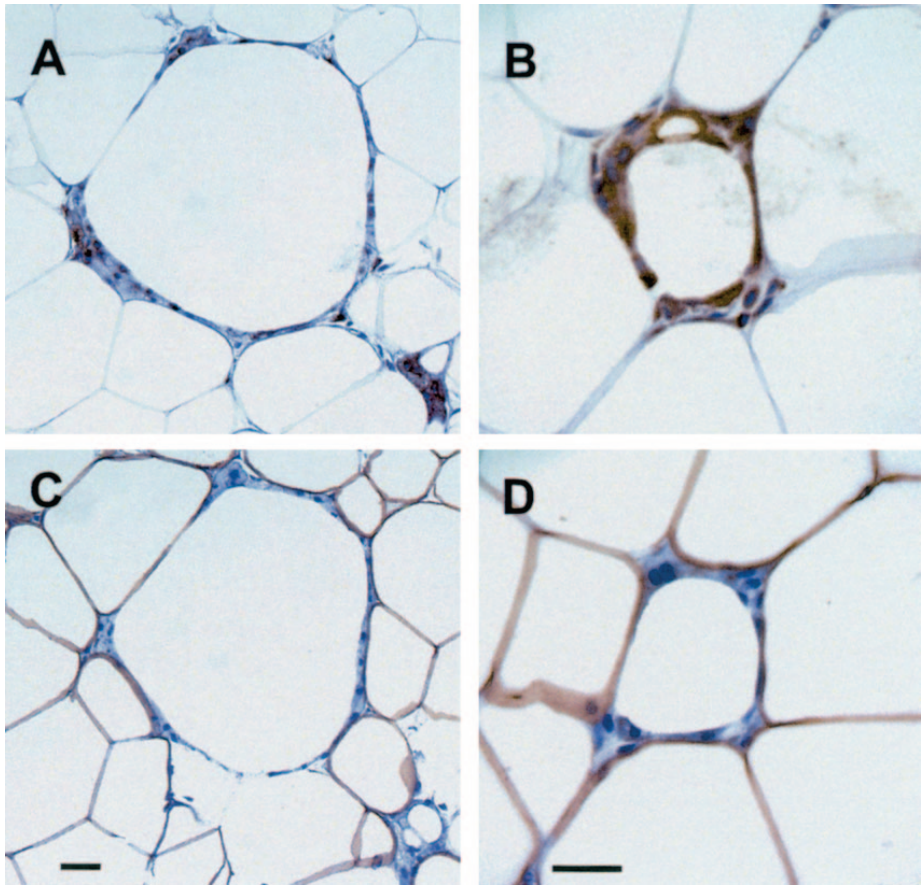

FIG. 4. Representative pictures showing crown-like structures in subcutaneous adipose tissue from an obese woman. Immunohistochemical staining for macrophage specific CD68 (*A* and *B*) is colored brown. *C* and *D*: Serial sections of *A* and *B*, respectively, were stained for perilipin (brown). All sections were counterstained with hematoxylin (colored blue). Scale bar: 20 μm.

the cellular origin of MCP-1, as several studies have consistently shown macrophages to be the main source of this chemokine in human adipose tissue (11,12,38). CCL3 (MIP-1α) is a monokine involved in the recruitment and activation of polymorphonuclear leukocytes (39). In the present study, PPAR-γ expression was significantly lower in the high than in the normal LFAT group. Ablation of adipocyte PPAR-γ expression in mice has been shown to lead to adipocyte necrosis and infiltration of inflammatory cells in adipose tissue (40). Therefore, the current observation of reduced adipose tissue PPAR-γ expression in the high LFAT group may have contributed to the greater levels of inflammation in the adipose tissue of these subjects.

Previous studies by Cancello et al. (13) and Cinti et al. (18) have demonstrated that the number of macrophages is increased in obese subjects compared with nonobese subjects. Furthermore, several studies have shown that macrophages form crown-like structures (11,12,38), which surround perilipin-negative adipocytes (18). The number of crown-like structures was increased in both obese mice and humans. The present data confirm the presence of these structures in human subcutaneous adipose tissue. The finding of increased macrophage infiltration independent of obesity is novel. The crown-like structures were found to surround perilipin-negative adipocytes. Perilipin, a protein involved in the regulation of lipolysis, is located at the interface between the cytosol and the triacylglycerol droplets of adipocytes (41). Cinti et al. (18) found negative perilipin staining in adipocytes, the membranes of which were disrupted and engulfed by macrophages when studied by electron microscopy. Negative perilipin staining was used as a marker of adipocyte death in the current study.

Fat cell size has been shown to correlate with obesity in multiple studies (42) and was also closely correlated with the degree of obesity in studies showing macrophage infiltration in adipose tissue (11). In a study by Cinti et al. (18), cell death correlated with mean adipocyte size. In $HSL^{-/-}$ mice, adipocyte cell size was markedly increased but body fat mass similar to that of wild-type mice. In these $HSL^{-/-}$ mice, adipose tissue was infiltrated with macrophages, implying that increased cell size rather than overall obesity is a trigger for macrophage infiltration (18). In the present study in equally obese groups, we found no difference in average fat cell size.

Regarding the mechanisms underlying the increase of ceramides, several factors could be involved. First, dietary

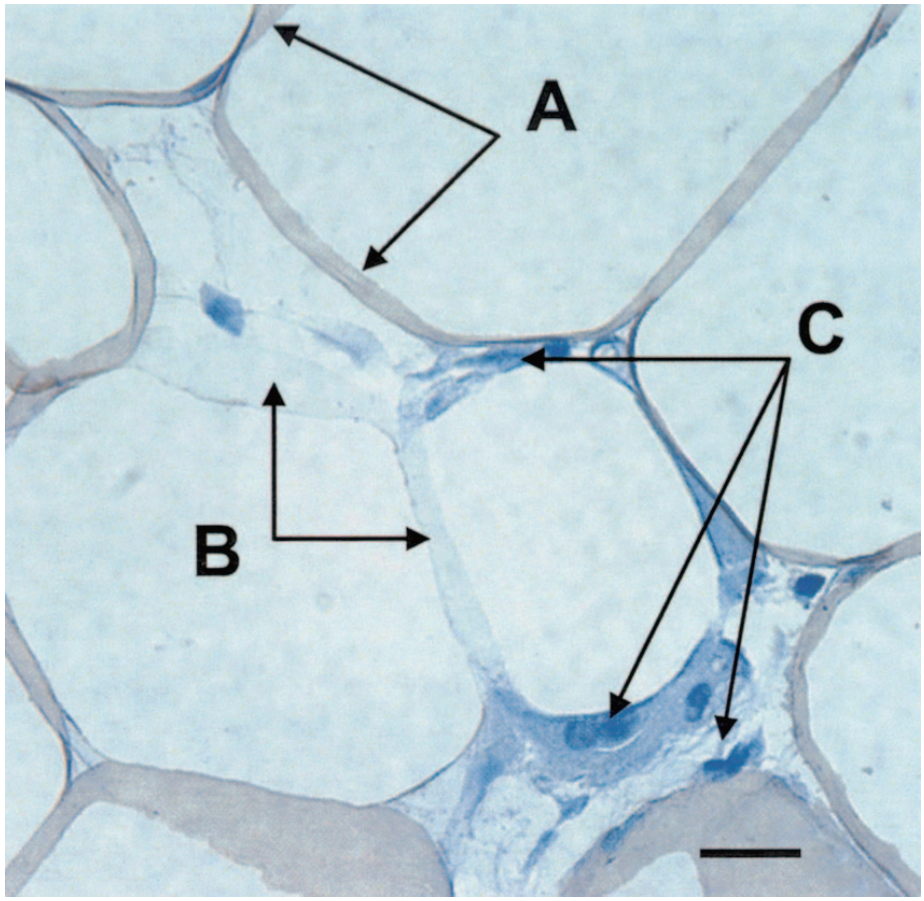

FIG. 5. Light microscopy of subcutaneous adipose tissue from an obese woman stained for perilipin (brown) showing dead (perilipin-free) adipocytes surrounded by macrophages (blue) forming a crown-like structure. The blue-colored nuclei (labeled C) stained positive for the macrophage-specific marker CD68 on a serial section (not shown). *A* arrows, perilipin-positive staining; *B* arrows, perilipin-negative staining; and *C* arrows, macrophages. Scale bar: 20 μm.

supplementation has been shown to increase plasma sphingomyelin concentrations (28). Sphingolipids are particularly abundant in dairy products, suggesting that their intake was greater in the high rather than the normal LFAT group. This theory is supported by the observation in the lipidomics analysis that the most upregulated triacylglycerol species contained odd numbers of total fatty acid carbons (Fig. 6*B*), implying that the concentration of odd-chain fatty acids was increased. Such fatty acids are likely to include fatty acids of exogenous origin specific for ruminant fat, namely 15:0 and 17:0, which are biological markers of dairy fat intake (43). These data are in agreement with previous data demonstrating that a relative high-fat intake correlates with increased LFAT both in type 2 diabetic patients (19) and in monozygotic twins discordant for obesity (20) and that LFAT content can be increased by a high-fat compared with low-fat diet in humans (44). Thus, ceramides could be mediators of the adverse metabolic effects of high-fat diets.

Increased de novo biosynthesis or hydrolysis of sphingomyelin via activation of sphingomyelinase(s) could also have contributed to the increase in ceramide content in the high LFAT group (28). The initial rate-limiting step of de novo biosynthesis of ceramides is strongly dependent on long-chain fatty acid availability (28), which was, based on the lipidomics analysis of adipose tissue, significantly increased in the high LFAT compared with the normal LFAT group. Finally, although enzymatic activities were not determined, expression levels of SMPD1 (acid sphingomyelinase) and SMPD3 (neutral sphingomyelinase 2) were significantly increased in the high compared with the normal LFAT group. Both SMPD1 and SMPD3 are increased by several cytokines including TNF-α (45). In the present study, TNF-α expression in adipose tissue was 1.6-fold greater in the high LFAT group, but this was not statistically significant ($P = 0.12$), possibly due to a type II error. Interestingly, sphingomyelinase expression is heavily increased in the arterial intima (46), and the ceramide content of LDL in the arterial wall is higher than that of native LDL in the plasma (47). Thus, the changes in inflamed adipose tissue containing macrophages bear some resemblance to changes characteristic of atherosclerosis in the arterial wall.

In conclusion, we demonstrated that adipose tissue is infiltrated with macrophages and that its content of long-chain triacylglycerols and ceramides is increased in sub-

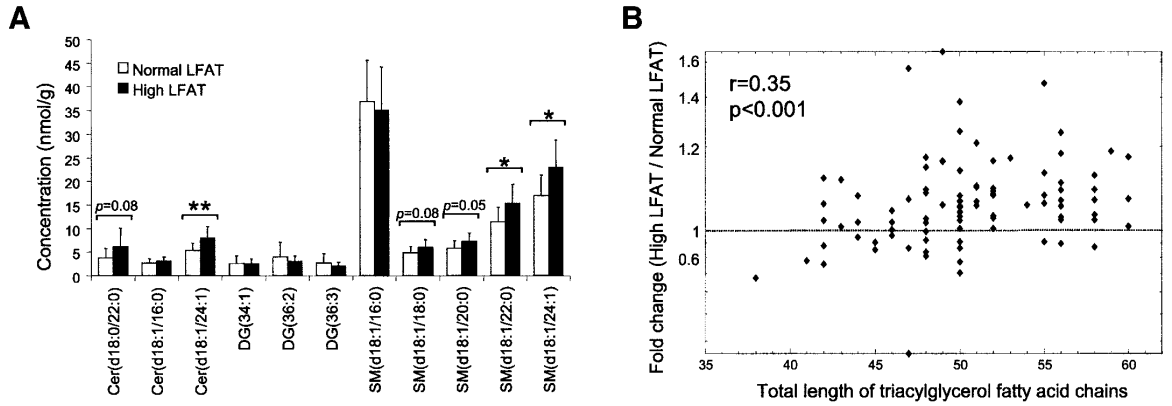

FIG. 6. Lipidomics profiling of subcutaneous adipose tissue. *A*: Levels of detected ceramide, diacylglycerol, and selected sphingomyelin species. *$P < 0.05$, **$P < 0.01$. *B*: Triacylglycerol carbon chain length correlates with the fold change between normal and high LFAT groups. Spearman correlation was applied to calculate r, with P value testing the hypothesis of no correlation against the alternative that there is a nonzero correlation using the large sample approximation.

jects with increased LFAT compared with equally obese subjects with normal LFAT content. Ceramides could mediate adverse effects of long-chain fatty acids and induce both insulin resistance and inflammation, although the present study does not prove cause and effect.

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Genetic factors contribute to variation in serum alanine aminotransferase activity independent of obesity and alcohol: A study in monozygotic and dizygotic twins☆

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***Background/Aims*: This study aimed to determine the heritability of serum alanine aminotransferase (S-ALT) and fasting serum insulin (fS-insulin) concentration as well as determine the association of these measures with liver fat content in young adult monozygotic (MZ) and dizygotic (DZ) twins.**

***Methods*: Three hundred and thirteen individual twins were recruited from a population-based cohort ($n = 4929$). The study subjects represented a wide range of body mass indexes (BMI), were free of any diseases or regular medications and had an intake of less than two drinks of alcohol/day. To verify that S-ALT is a marker of liver fat, it was measured by proton magnetic resonance spectroscopy (^{1}H MRS) in 66 subjects. Heritability estimations were performed using BMI- and gender-adjusted values.**

***Results*: Intra-pair correlations were significantly higher in the MZ twins than the DZ twins for both S-ALT (0.65 for MZ and 0.04 for DZ) and fS-insulin (0.58 and 0.34, respectively). Heritability of S-ALT was 55% and that of fS-insulin 61%. In the 66 subjects S-ALT ($r = 0.70$ for women and $r = 0.50$ for men, $p \leqslant 0.01$ for both) and fS-insulin ($r = 0.58$ and $r = 0.59$, respectively, $p \leqslant 0.01$ for both) concentrations correlated significantly with liver fat content.**

***Conclusions*: These twin data suggest that approximately 60% of the variation in S-ALT, a marker of liver fat content, is genetically determined.**

***Keywords*: Heritability; ALT; Insulin; Liver fat; NAFLD; Twins**

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Abbreviations: AIC, Akaike Information Criterion; BMI, body mass index; CI, confidence interval; DEXA, dual-energy X-ray absorptiometry; d.f., degrees of freedom; DZ, dizygotic; fS-, fasting serum; fP-, fasting plasma; ^{1}H MRS, proton magnetic resonance spectroscopy; MRI, magnetic resonance imaging; MZ, monozygotic; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; S-ALT, serum alanine aminotransferase; S-AST, serum aspartate aminotransferase; S-GGT, serum gamma glutamyl transferase; SNP, single nucleotide polymorphism.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of steatosis and elevated liver enzymes in the US [1]. NAFLD is defined as fat accumulation in the liver exceeding 5–10% by weight in subjects who do not consume significant amounts of alcohol and who do not have clinical or laboratory evidence of autoimmune, viral or toxin-induced liver disease, or of inborn errors of metabolism [2]. An increase in liver fat due to NAFLD is an integral feature of the metabolic syndrome and closely correlated with all its components; central obesity, insulin resistance, dyslipidemia and hypertension [3].

Liver fat and serum alanine aminotransferase (S-ALT) correlate with body mass index (BMI), but the relationship is weak [4]. Thus, at any given BMI, liver fat content and S-ALT vary considerably. In lipodystrophic patients, liver fat is increased despite an almost complete lack of subcutaneous fat [5]. The causes of interindividual variation in liver fat content independent of BMI and obesity are unclear. There are almost no data regarding the possible contribution of genetic factors to variation in liver fat content. Family clustering [6,7], interethnic variation [8,9] and single nucleotide polymorphism (SNP) studies (e.g. genes involved in lipid metabolism, inflammation, oxidative stress and iron metabolism) suggest that genetic factors may be important in determining steatosis [10–16] and non-alcoholic steatohepatitis (NASH) [17–26].

In this study, we estimated the relative roles of genetic and environmental influences on inter-individual variation in S-ALT and fasting serum insulin (fS-insulin) concentrations in young adult monozygotic (MZ) and dizygotic (DZ) twins. The relationship between S-ALT and fS-insulin and directly measured (proton magnetic resonance spectroscopy, ^{1}H MRS) liver fat content was determined in a subset of 66 subjects.

2. Patients and methods

2.1. Subjects and study design

The participants were recruited from a population-based longitudinal study (FinnTwin16) of five consecutive birth cohorts (1975–1979) of twins ($n = 4929$ individuals), their siblings and parents, identified through the national population registry of Finland [27]. The twins had been studied by questionnaires at 16, 17, 18.5 and 23–27 years of age. The present study subjects were same-sex pairs enrolled based on their responses to questions on weight and height at the last follow-up, with the aim to cover the full BMI range of both normal-weight and obese subjects. The present sample comprised of MZ ($n = 120$ twins, 57 full pairs, 36 female and 21 male pairs) and same-sex DZ ($n = 193$ twins, 88 pairs, 50 female and 38 male pairs) twins. The mean ± SE BMI of the study sample, based on self-reported data was 24.1 ± 0.2 kg/m^2, range 17.6–42.9 kg/m^2, which is comparable with the whole cohort (22.9 ± 0.1 kg/m^2, range 14.0–44.2 kg/m^2). Exclusion criteria included (1) any known acute or chronic disease other than obesity based on medical history and physical examination, electrocardiogram and standard laboratory tests (blood counts, serum creatinine, serum thyroid stimulating hormone (S-TSH) and electrolyte concentrations), (2) pregnancy and lactation (in women), (3) clinical signs or symptoms of hepatitis or inborn errors of metabolism, (4) a history of use of toxins or drugs associated with liver steatosis, (5) regular medications other than oral contraceptives, (6) self-reported alcohol consumption more than 2 drinks (24 g of alcohol) per day [28] (daily average of a four-week period by questionnaire, 39 individuals were excluded because of excess drinking) and (7) unstable weight (±5 kg) for the past three months. Among female subjects, 12 women had given birth to one child, five to two children and two to three children. Zygosity was confirmed by genotyping of ten informative genetic markers [29]. All pairs were Caucasian, and their mean age was 27.3 ± 0.2 years (range 23.2–32.2).

Each subject was studied after an overnight fast of 10–12 h. Blood samples were taken for screening purposes as detailed above and for measurements of S-ALT, serum aspartate aminotransferase activity (S-AST), serum gamma glutamyl transferase activity (S-GGT) and fS-insulin and fasting plasma glucose (fP-glucose) concentrations. Medical history was reviewed and physical examination was performed, and weight and height were measured barefoot in light clothing to calculate BMI (kg/m^2). Percent body fat was measured by using dual-energy X-ray absorptiometry (DEXA) (Lunar Prodigy, software version 2.15, Madison, WI) [30]. To validate the use of S-ALT as a marker of liver fat content in the present study, liver fat was measured by ^{1}H MRS in a subgroup of 66 subjects (46 MZ and 20 DZ subjects) with BMIs ranging from 19.4 kg/m^2 to 45.8 kg/m^2. We also analysed how liver fat content differs between MZ twins discordant for obesity ($n = 13$ twin pairs, 6 female and 7 male pairs) using a previously characterized subgroup [29,31–35], whose intra-pair BMI differences were on the average 5.0 ± 0.5 kg/m^2. Informed consent was obtained from each patient included in the study. The protocol was designed and performed according to the principles of the Helsinki Declaration and was approved by the Ethical Committee of the Helsinki University Central Hospital.

2.2. Liver fat content (^{1}H MRS)

The liver fat content was determined using proton magnetic resonance spectroscopy as previously described [4].

2.3. Analytical procedures

fP-Glucose concentrations were measured in duplicate with the glucose oxidase method using Glucose Analyzer II (Beckman Instruments, Fullerton, CA) [36], fS-insulin with radioimmunoassay (Phadeseph Insulin RIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), and S-ALT, S-AST and S-GGT activities as recommended by the European Committee for Clinical Laboratory Standards.

2.4. Statistical analyses

Non-normally distributed data were used after logarithmic transformation. For individual twins, the statistical analyses, significance tests and 95% confidence intervals (95% CI) were corrected for clustered sampling of co-twins within pairs by using survey methods [37]. Pearson's correlation coefficients were calculated to determine associations between liver fat, liver enzymes, fS-insulin and BMI. Analysis of covariance was used to compare slopes and intercepts of regression lines between liver fat vs. S-ALT and liver fat vs. fS-insulin for men and women. If neither slopes nor intercepts differed between women and men, a common regression equation was calculated for all data. Wald test (T-test adapted for clustered twin data) for independent samples was used to compare males and females, and MZ and DZ twins. Twin similarity within each zygosity group was assessed using intra-pair correlations to provide initial evidence for familial aggregation and the presence of genetic effects. These statistical analyses were performed using the Stata statistical software (release 9.0; Stata Corporation, College Station, TX) and GraphPad Prism (version 4.00 for

Windows; GraphPad Software, San Diego, CA). A p-value less than 0.05 was considered statistically significant. Data are means ± SE unless otherwise indicated.

2.5. Heritability analyses

The intra-pair correlations and heritability estimates were calculated for both genders together using gender-adjusted variables. Because of high correlation between BMI and S-ALT ($r = 0.44$) and BMI and fS-insulin ($r = 0.56$), BMI-adjusted values of S-ALT and fS-insulin were used. Thus, the heritability estimates for S-ALT and fS-insulin were independent of gender and BMI. Quantification of the genetic and environmental effects on S-ALT and fS-insulin concentrations was performed using the Mx statistical package (6th ed., Richmond, VA) [38,39].

The genetic variation can be divided into additive (A) genetic effects of individual alleles (correlation 1.0 for MZ and 0.5 for same-sex DZ co-twins) and to dominant (D) genetic effects by allelic interactions within a loci (correlation 1.0 for MZ and 0.25 for same-sex DZ co-twins). The environmental variation can be attributed to common (C) environmental effects shared by the co-twins (by definition, correlation 1.0 for both MZ and DZ co-twins) and unique, individual-specific (non-shared), environmental (E) effects (uncorrelated between the co-twins). One can fit models based on different combinations of these parameters: ADE, ACE, AE, DE, CE and E. Our data include only twins reared together and do therefore not allow modeling of genetic dominance and common environmental effects simultaneously. In classical twin data, possible gene–environment interaction effects are estimated as part of additive genetic component, which thus may also reflect genetic differences in susceptibility to environmental factors. Further, we need to make the assumptions of random mating which, if not present, would increase DZ correlations and thus inflate estimates of common environmental variance and reduce genetic variance [40].

The primary objective in heritability model fitting is to explain the observed data by finding a model that best balances parsimony and goodness of fit with as few parameters as possible [39]. The model fitting starts with full ADE or ACE models, to which the fit of the submodels AE, DE, CE and E may be compared by using chi-square difference test statistics and degrees of freedom (d.f.) between nested models. The Akaike Information Criterion (AIC) is a global test of model fit combining the chi-square value and d.f.; negative values of the AIC are considered to provide evidence for a better fit. From the best fitting model, it is possible to estimate the proportion of total variance attributable to A, D, C and E. Bivariate Cholesky decomposition parameterization [39] was calculated to determine whether the S-ALT, fS-insulin and BMI shared genetic or environmental effects. This was estimated by correlations between genetic effects (r_a) and environmental effects (r_c, r_e) for the two measures.

3. Results

3.1. Subject characteristics

3.1.1. Women and men

The study sample of 313 subjects comprised 178 women and 135 men. Women and men were similar with respect to BMI (24.3 ± 0.5 vs. 25.3 ± 0.4 kg/m^2, women vs. men, $p = 0.09$) and liver fat (4.7 ± 1.7 vs. 6.8 ± 2.4%, respectively, $p = 0.46$). fS-insulin concentrations were also comparable (6.1 ± 0.3 vs. 6.7 ± 0.5 mU/l, $p = 0.30$), while S-ALT (20 ± 2 vs. 37 ± 2 U/l, $p < 0.001$), S-AST (25 ± 1 vs. 29 ± 1 U/l, $p < 0.001$) and S-GGT (18 ± 1 vs. 28 ± 2 U/l, $p < 0.001$) concentrations were significantly lower in women than in men.

3.1.2. MZ and DZ twins (Table 1)

A total of 120 MZ twins (57 full pairs) and 193 DZ twins (88 full pairs) were studied. There were no differences in mean variances of the physical and biochemical characteristics between MZ and DZ twins (Table 1), and thus the assumption of trait similarity between MZ and DZ twins for twin modeling was met. As expected, within-pair differences in BMI were greater in the DZ (4.6 ± 0.4 kg/m^2, range 0.0–15.2 kg/m^2) than in the MZ twins (2.7 ± 0.4 kg/m^2, range 0.1–10.1 kg/m^2, $p < 0.001$).

3.2. Relationships between S-ALT and fS-insulin concentrations, and liver fat content

S-ALT was strongly positively correlated with liver fat content in both women ($r = 0.70$, 95% CI 0.62–0.77, $p = 0.002$) and men ($r = 0.50$, 95% CI 0.36–0.62, $p = 0.01$) (Fig. 1). A strong positive correlation was also found between liver fat content and fS-insulin concentration. This was similar in both genders ($r = 0.59$, 95% CI 0.40–0.73, $p < 0.001$) (Fig. 1). BMI-adjusted correlation between S-ALT and liver fat was 0.42 (95% CI 0.32–0.51, $p < 0.001$) and between fS-insulin and liver fat 0.37 (95% CI 0.27–0.46, $p = 0.006$). The correlation between liver fat content and BMI was 0.47 (95% CI 0.26–0.64, $p = 0.057$).

3.3. Intra-pair correlations for S-ALT and fS-insulin concentrations

Assessments of within-pair similarity were performed by using BMI- and gender-adjusted measures. Results were similar if the percent body fat was used instead of BMI for the adjustments (data not shown). The intra-pair correlations (twin A vs. twin B) for S-ALT were significantly higher ($p < 0.001$ for the difference between MZ and DZ correlation coefficients) for MZ ($r = 0.65$, 95% CI 0.50–0.80, $p < 0.001$) than for DZ twin pairs ($r = 0.04$, 95% CI 0.0–0.25, $p = 0.37$) (Fig. 2), demonstrating the influence of genetic factors. For fS-insulin, the intra-pair correlations tended to be higher ($p = 0.076$) for MZ ($r = 0.58$, 95% CI 0.40–0.76, $p < 0.001$) than for DZ ($r = 0.34$, 95% CI 0.15–0.53, $p < 0.001$) pairs (Fig. 2). For S-ALT, the DZ correlations were less than half of the MZ correlations, suggesting that not only additive genetic but also possibly dominant genetic factors influence the trait. For fS-insulin, the DZ correlations were more than half of the MZ correlations, suggesting the presence of shared environmental effects (e.g. within the family). Thus, we used the ADE model for S-ALT

Table 1
Physical and biochemical characteristics of the MZ and DZ twins.

Variable	MZ	DZ	*p*-Value
Number of twins	120 (57 full pairs)	193 (88 full pairs)	
Age (years)	27.1 ± 0.3	27.4 ± 0.2	0.38
Body mass index (kg/m^2)	25.3 ± 0.6	24.4 ± 0.3	0.20
Body fat (%)	30.0 ± 1.3	28.8 ± 0.9	0.43
fS-ALT (U/l)	27.2 ± 2.5	27.4 ± 1.8	0.95
fS-AST (U/l)	27.1 ± 1.2	26.5 ± 0.9	0.71
fS-GGT (U/l)	21.0 ± 1.9	22.8 ± 1.7	0.48
fP-Glucose (mmol/l)	5.0 ± 0.1	4.9 ± 0.04	0.18
fS-Insulin (mU/l)[a]	6.3 ± 0.4	6.3 ± 0.4	0.98

Data are means ± SE, *p*-value is calculated using Wald test for equality of means in MZ and DZ twins.
MZ, monozygotic; DZ, dizygotic; fS-, fasting serum; fP-, fasting plasma; ALT, alanine aminotransferase activity; AST, aspartate aminotransferase activity; GGT, gamma glutamyl transferase activity.
[a] $n = 114$ MZ, 187 DZ.

and the ACE model for fS-insulin as starting point in heritability analyses.

3.4. Heritability analyses for S-ALT and fS-insulin concentrations

The heritability estimates were analysed using the combined data of both genders with gender- and BMI-adjusted values. For S-ALT, the ADE model fitted the data slightly better than the AE model (chi-square change $p = 0.035$). For both models, AIC was low, implying good fit (AIC = −711.64 for the AE and −714.06 for the ADE model). In the ADE model, all of the genetic influence was placed on the D effect. Since dominant effects are rare in the absence of additive effects, the AE model was chosen as the final model for S-ALT.

For fS-insulin, the ACE model was used initially. The AIC was higher in the ACE (AIC = −664.87) model than in the AE model (AIC = −666.87). Dropping the C effect from the ACE model did not worsen the fit ($p = 0.97$), and the point estimate for C effects in the ACE model was small and non-significant. This implies that shared family environmental effects for fS-insulin are not significant and that the AE model fit the data the best.

AE models were used in the subsequent analyses. Next, three Cholesky bivariate decomposition analyses were performed to analyze the contributions of genetic and environmental effects and their correlations on S-ALT, fS-insulin and BMI. First, in a model where S-ALT and fS-insulin were adjusted for BMI and gender, genetic effects explained 55% (95% CI 36–70%) of the variation of S-ALT and 61% (95% CI 42–73%) of that of fS-insulin. The contribution of environmental effects was 45% (95% CI 30–64%) for S-ALT and 39%

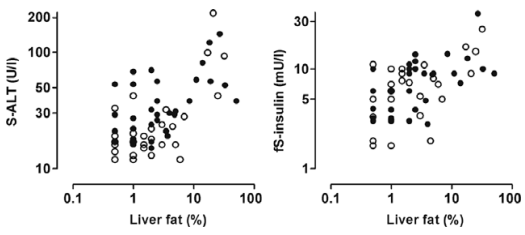

Fig. 1. The relationship between liver fat content (measured using ^{1}H MRS) and S-ALT (log scales) ($r = 0.70$, $p = 0.002$ for women; $r = 0.50$, $p = 0.01$, for men) and the relationship between liver fat content and fS-insulin concentration (log scales) ($r = 0.59$, $p < 0.001$ for both genders). Open circles denote women ($n = 32$) and filled circles denote men ($n = 34$). ^{1}H MRS, proton magnetic resonance spectroscopy; S-ALT, serum alanine aminotransferase activity; fS-, fasting serum.

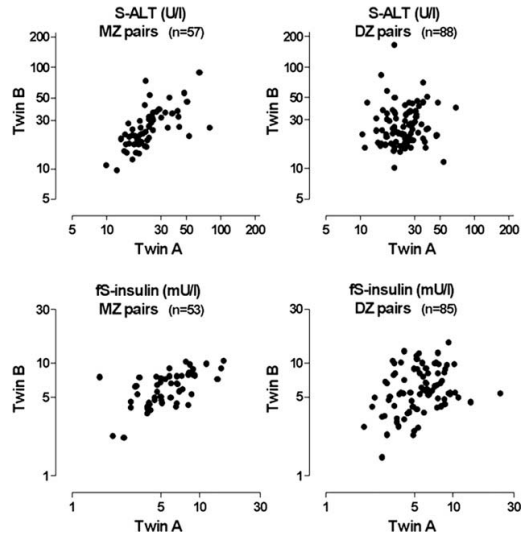

Fig. 2. Intra-pair correlations between twin A and twin B in MZ and DZ twins for BMI- and gender-adjusted S-ALT (log scale) ($r = 0.65$, $p < 0.001$ for MZ; $r = 0.04$, $p = 0.37$ for DZ) and for BMI- and gender-adjusted fS-insulin concentrations (log scale) ($r = 0.58$, $p < 0.001$ for MZ; $r = 0.34$, $p = 0.001$ for DZ). MZ, monozygotic; DZ, dizygotic; S-ALT, serum alanine aminotransferase activity; fS-, fasting serum.

(95% CI 27–58%) for fS-insulin. Bivariate genetic modeling revealed that the correlation between additive genetic effects on S-ALT and fS-insulin was 0.30 (95% CI 0.09–0.51), implying that 9% (0.30×0.30) of the genetic factors influencing variation in S-ALT and fS-insulin concentrations are the same. No shared environmental effects explained the correlation between S-ALT and fS-insulin when BMI was adjusted in the model.

Second, two bivariate models for S-ALT and BMI, and for fS-insulin and BMI, were estimated. In the bivariate model for S-ALT and BMI (adjusted for gender), the genetic correlation between these two measures was 0.39 (95% CI 0.12–0.58) and the environmental correlation 0.51 (95% CI 0.30–0.68). In the bivariate model for fS-insulin and BMI, the genetic correlation (adjusted for gender) was 0.53 (95% CI 0.31–0.68) and the environmental correlation 0.64 (95% CI 0.46–0.77). This implies that both S-ALT and fS-insulin share both genetic and environmental factors with BMI. The heritability for BMI was 68% in these models and, as mentioned above, 55% for S-ALT and 61% for fS-insulin.

3.5. Genetic and lifestyle effects on liver fat content

In the 23 MZ pairs with liver fat measures, the intra-pair correlation for liver fat content was high ($r = 0.70$, 95% CI 0.49–0.92, $p < 0.001$), but the number of DZ pairs with liver fat measures was too small to permit reliable heritability model fitting directly for liver fat content. Evidence of non-genetic effects was obtained from a correlation between intra-pair differences of liver fat and those of BMI ($r = 0.47$, 95% CI 0.07–0.74, $p = 0.009$), which implied that BMI differences explained 22% (0.47×0.47) of the differences in liver fat within MZ pairs.

In the 13 obesity-discordant MZ twin pairs (BMI difference 5.0 ± 0.5 kg/m^2), the obese MZ co-twins had significantly higher ($p = 0.002$ for the difference between obese MZ and non-obese MZ co-twins) liver fat content ($7.3 \pm 2.1\%$) than the non-obese MZ co-twins ($2.0 \pm 0.4\%$). The effects of acquired obesity on liver fat content varied considerably between the twin pairs, and this was not explained by BMI differences or by gender (Fig. 3).

4. Discussion

In this study, we estimated the heritabilities of S-ALT and fS-insulin concentrations as markers of liver fat content in 313 young adult MZ and DZ twins. Heritability was high and significant both for S-ALT (55%) and for fS-insulin (61%). To validate the use of S-ALT as marker of liver fat, we measured liver fat content by ^{1}H MRS in a subgroup of 66 subjects. In keeping with our previous findings [3,41], we found positive correlations between liver fat content and S-ALT ($r = 0.50$–0.70) and fS-insulin (0.59). The high intra-pair correlation for liver fat content in MZ pairs ($r = 0.70$) was consistent with these high heritability estimates for S-ALT and fS-insulin.

The 313 subjects were recruited from a large population-based twin cohort comprising of 4929 Finnish individual twins. The study sample covers a wide range of BMI comparable with the whole cohort and represents healthy young adults who do not use regular medications. Medical history and daily alcohol consumption were carefully reviewed to exclude causes of steatosis and elevated liver enzymes other than those associated with obesity and insulin resistance. This enabled reliable estimation of genetic causes in variation of S-ALT and fS-insulin concentrations. Furthermore, MZ and same sex DZ twins are perfectly matched for age, sex and ethnicity.

The heritability of liver function tests has previously been estimated in two twin studies [42,43]. Bathum et al. investigated 290 pairs of elderly (age range 73–102 years) Danish same sex twins and found 35–61% heritability for S-ALT, S-GGT, serum bilirubin, serum albumin and serum lactate dehydrogenase [42]. The heritability for S-ALT was 35% and the best-fitting model for S-ALT was DE (35% for dominant genetic and 65% for unique environmental effects). The results did not change after adjusting for self-reported BMI and alcohol consumption. Information regarding medical history or medication status were not included in the analyses. In the present study we found almost a twice as great estimate of heritability of S-ALT concentration than in the Danish study [42]. In contrast to this study, the Danish twins were old, their disease records or medications were not considered and BMI was self-reported.

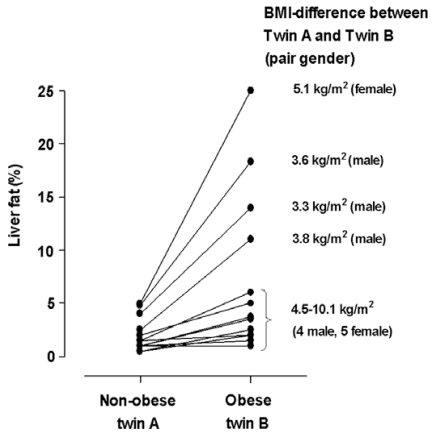

Fig. 3. Effects of acquired obesity on liver fat content (measured using ^{1}H MRS) in 13 obesity-discordant MZ twin pairs. ^{1}H MRS, proton magnetic resonance spectroscopy; MZ, monozygotic.

These factors can introduce variability that tends to decrease twin similarity and heritability estimations.

Notably, both the present and the Danish study suggested that genetic effects due to dominance (i.e. the interactions between alleles of the relevant genes rather than simple additive effects alone) contribute to variation in S-ALT. However, the twin model without information on other family relationships, has poor power to distinguish between additive and dominant genetic effects. Therefore both the present and the Danish studies provide evidence of substantial genetic influences, but do not permit us to unequivocally distinguish between additive and dominant effects.

Middelberg et al. studied 965 Australian families with adolescent twins and their non-twin siblings to investigate the contribution of genetic and environmental factors to variation in S-ALT, S-AST, S-GGT and serum uric acid concentrations [43]. Genetic and environmental factors (e.g. diet, lifestyle) influencing these four parameters were studied longitudinally at ages of 12, 14 and 16 years in the same twins. The heritability of S-ALT was 40%. The contribution of obesity, alcohol consumption, medications or disease records to heritability of S-ALT was not reported in the Australian study [43].

Previous studies have shown that fS-insulin is a good marker for liver fat content, even independent of age, gender and BMI [3]. The fatty liver is insulin resistant [44–46] which impairs the ability of insulin to inhibit hepatic glucose production resulting in mild hyperglycemia and hyperinsulinemia. Impaired hepatic insulin clearance also contributes to hyperinsulinemia in subjects with a fatty liver [46,47]. Consistent with these data we found close correlations between liver fat content and fS-insulin concentration. The relationship was similar in both men and women. The heritabilities for S-ALT (55%) and fS-insulin (61%) concentrations were of similar magnitude. These results suggest that genetic factors underlying interindividual differences in liver fat content may also regulate fS-insulin concentrations e.g. by changing insulin clearance and action or vice versa. Nonetheless only a small fraction (9%) of the genetic effects underlying S-ALT were shared with those genetic effects influencing fS-insulin concentration, indicating that these two markers for liver fat content are mainly determined by separate sets of genes.

Although liver fat content varies considerably at any given BMI [4], it is also regulated by acquired factors, especially changes in body weight [31]. However, whether there is interindividual variation in the response of liver fat accumulation to changes in body weight has not been examined. In this study we analyzed a rare subgroup of young MZ twin pairs discordant for obesity. This analysis suggested that the tendency to deposit fat in the liver in acquired obesity is not simply explained by BMI. This result would be consistent with the seminal work on MZ twins by Bouchard et al., who showed that changes in the amount and distribution of fat (subcutaneous/visceral) in response to overfeeding and negative energy balance seem to be largely explained by genetic factors [48]. However, since our subjects were young and had relatively low liver fat contents, our conclusions may not necessarily translate to older age groups with higher liver fat contents. With advanced age, the contribution of environmental and lifestyle factors may become more evident.

In conclusion, our data in healthy young adult twins suggest that genetic factors contribute to variation in S-ALT, a marker of liver fat content. Detailed characteristics of these genetic factors will help understand why some individuals, but not others, accumulate fat in the liver and develop metabolic syndrome.

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